STABLE ISOTOPES AND METABOLITE PROFILES AS PHYSIOLOGICAL MARKERS FOR THE DROUGHT STRESS SENSITIVITY IN DOUGLAS-FIR PROVENANCES (*Pseudotsuga menziesii* (MIRB.) FRANCO)

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"GOING TO THE WOODS IS GOING HOME."

- John Muir

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Abbreviations

^{13}C	carbon-13, the stable isotope of carbon with a nucleus containing six protons and seven neutrons
^{18}O	oxygen-18, the stable isotope of oxygen with a nucleus containing eight protons and ten neutrons
a	carbon isotope fractionation during diffusion of CO_2 through the stomata
$a\Delta_R$	Apparent respiratory fractionation
AHM	Long-term annual heat:moisture index (=(MAT+10)/(MAP/1000))
A_n	photosynthetic assimilation capacity (net CO_2 assimilation rate per unit leaf area)
A_{max}	maximum photosynthetic assimilation capacity
ANCOVA	analysis of covariance
ANOVA	analysis of variance
b	carbon isotope fractionation during carboxylation by Rubisco
B	bark
BC	British Columbia, Province in Canada
C	control
c_a	CO_2 concentration in ambient air
c_c	CO_2 concentration at sites of carboxylation
c_i	CO_2 concentration in leaf intercellular air spaces
CMD	long-term Hargreaves climatic moisture deficit (=sum of the monthly difference between a reference evaporation and precipitation)
CoA	coenzyme A
CR	coarse roots
d	day(s)
d100	diameter at breast height (1.3 m) of the mean basal area tree of the 100 thickest trees per hectar
DAT	days after treatment start
dbh	diameter at breast height
e_a	water vapour pressure in the atmosphere
e_i	water vapour pressure in the leaf intercellular air space
FR	fine roots
GC-MS	gas chromatography–mass spectrometry
g_m	mesophyll conductance to CO_2
g_s	stomatal conductance to H_2O
GS	growing season
h100	height of the mean basal area tree of the 100 thickest trees per hectar
IWUE	intrinsic water-use efficiency
$IWUE_{13C}$	IWUE derived from stable isotope composition in needle WSOM
$IWUE_{13C\ TOM}$	IWUE derived from $\delta^{13}C$ in TOM of current-year needles sampled in September of the respective year, integrating over one growing season
$IWUE_{13C\ WSOM\ AVG}$	IWUE derived from $\delta^{13}C$ in previous-year needle WSOM averaged over the measurement period
$IWUE_{GE}$	IWUE derived from gas exchange measurements as the ratio between A_n and g_s
$IWUE_{GE\ AVG}$	$IWUE_{GE}$ averaged over the measurement period
$IWUE_{GE\ slope}$	slope of the regression line of all measured A_n versus all measured g_s, as an IWUE integrating over the whole measurement period
m asl	meter above sea level

MAP, MAT	long-term average annual precipitation sum and temperature, respectively
MC	Monte Creek, a Douglas-fir provenance from BC, Canada
MGSP, MGST	long-term average growing season (April to Sep) precipitation sum and temperature, respectively
MSP, MST	long-term mean summer (May to Sep) precipitation and temperature, respectively
N	number of individuals
N08	needles developed in 2008
N09	previous-year needles from 2009
N10	current year's needles from 2010
OA	osmotic adjustment
OR	Oregon, State in USA
PO	Pend Oreille, a Douglas-fir provenance from WA, USA
PPFD	photosynthetically active photon flux density
P_{sum}	sum of precipitation (year, GS (growing season), April–Sep, Jun–Jul, June, July, Aug indicate the periods the climatic parameters were summed up or averaged over for a given year)
R	Pearson's correlation coefficient
ROS	reactive oxygen species
SD	standard deviation
SE	standard error
SH	sunshine hours
SHM	long-term summer heat:moisture index (=mean warmest month temperature/(MSP/1000))
T	treatment
TCA	tricarboxylic acid
T_{max}	average daily maximum temperature (year, GS (growing season, April–Sep), Jun–Jul, June, July, Aug indicate the periods the climatic parameters were summed up or averaged over for a given year)
T_{mean}	average of daily mean temperatures (year, GS (growing season, April–Sep), Jun–Jul, June, July, Aug indicate the periods the climatic parameters were summed up or averaged over for a given year)
T_{min}	average of daily minimum temperature (year, GS (growing season, April–Sep), Jun–Jul, June, July, Aug indicate the periods the climatic parameters were summed up or averaged over for a given year)
TOM	total organic matter
VPD	vapour pressure deficit
WA	Washington State, USA
WSOM	water-soluble organic matter
$\bar{x}$	mean height
$\Delta^{13}C$	discrimination against ^{13}C during photosynthesis
$\Delta^{18}O$	^{18}O enrichment
$\delta^{13}C_{atm}$	carbon isotope composition of atmospheric air
$\delta^{13}C_{plant}$	relative deviation of $^{13}C/^{12}C$ of the plant sample from $^{13}C/^{12}C$ of PDB ($\delta^{13}C_{PDB} = 0$)
$\delta^{13}C_R$	$\delta^{13}C$ in canopy respired CO_2
$\delta^{13}C_{TOM}$	carbon isotope composition of leaf total organic matter
$\delta^{13}C_{WSOM}$	carbon isotope composition of leaf water-soluble organic matter
$\delta^{18}O$	relative deviation of $^{18}O/^{16}O$ of the sample from $^{18}O/^{16}O$ of VSMOW ($\delta^{18}O_{VSMOW} = 0$)
$\delta^{18}O_{TOM}$	oxygen isotope composition of leaf total organic matter
$[CO_2]$	carbon dioxide concentration

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Summary

Summer droughts and periods of strongly increased temperature are expected to be more frequent in the future with potentially detrimental effects on trees and forests in Central Europe. Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is often discussed as an alternative for the drought and heat sensitive Norway spruce (*Picea abies* (L.) H.Karst.), which is one of the most important timber species in Central Europe but might be at risk in the future climate. The natural range of Douglas-fir in North America extends from north to south over up to 4,500 km. Two varieties with genetically diverse populations offer great potential to identify productive and drought-tolerant Douglas-fir provenances. This study aimed at studying the drought response of various Douglas-fir provenances linking phenotypic and physiological variation to elucidate mechanisms underlying drought sensitivity and resistance.

We took advantage of a provenance trial in south-western Germany located along an elevation gradient to study 50 year old Douglas-fir trees (ca. 25 - 35 m tall) of differing provenances. Furthermore, we conducted an experiment under controlled conditions to simulate heat wave effects on saplings of two provenances. We analysed leaf isotopic composition ($\delta^{13}C$ and $\delta^{18}O$), leaf gas exchange, metabolic changes and tree growth under heat and drought stress.

An early response to water deficit involves the regulation of stomatal conductance to water vapour that at the same time reduces CO_2 supply for assimilation rate. Changes in the intrinsic water-use efficiency can be assessed as i) the ratio between stomatal conductance and assimilation rate derived from instantaneous gas exchange measurements on the leaf, and by ii) stable carbon isotope composition ($\delta^{13}C$) of plant organic matter. Our results show concurring results for the intrinsic water-use efficiency derived from instantaneous gas exchange measurements on the leaf and $\delta^{13}C$ of leaf water-soluble organic matter (representing recent assimilates) when applied on trees growing at a humid site. 50-years old Douglas-fir trees growing under generally wet conditions were more responsive to drought in terms of their stomatal regulation than trees growing at a dry valley site. The information derived from leaf gas exchange measurements and $\delta^{13}C$ was not closely related to each other at the dry site. Enhanced osmotic adjustment under dry conditions sustained leaf turgor and stomatal conductance, and thus explains the low sensitivity of gas exchange measurements and $\delta^{13}C$ to changing environmental conditions observed at this field site. When assessing physiological responses on

the leaf level, we need to take into account i) the interaction between osmotic regulation and leaf physiological parameters assessed by leaf gas exchange measurements and $\delta^{13}C$, and ii) the acclimation of trees growing for decades at specific site conditions leading to strong differences in their drought responses.

Tree ring analyses revealed that the radial growth decline during the severe drought in 2003 could be clearly attributed to a reduction in stomatal conductance as assessed by the relations between $\delta^{18}O$ and $\delta^{13}C$ in the tree rings. A strong response became also apparent with instantaneous gas exchange measurements: From the humid to very dry conditions, a strong decline in stomatal conductance was observed. The provenance with the strongest stomatal response was Conrad Creek, both in the tree ring study and during the instantaneous gas exchange measurements, indicating concurrent results in instantaneous and retrospective methods.

Our isotopic analyses on current-year needle total organic matter showed a strong relation to weather conditions during the summer months, pointing to storage pools strongly affecting the total organic carbon in Douglas fir needles. During extreme drought, tree ring archives do not necessarily provide information due to growth cessation, rendering current-year needles a reliable alternative source for physiological information on recent summer months.

Stomatal closure decreases the leaf internal carbon dioxide concentration and impairs photosynthesis. The consequence is not only reduced growth but also the risk that light energy cannot be quenched photochemically and might support the formation of cytotoxic reactive oxygen species. Photoprotective mechanisms such as non-photochemical quenching (NPQ), scavenging of reactive oxygen species and production of volatiles avoid damage from excessive light energy. We found high levels of NPQ in provenances with a strong stomatal response to drought whereas a low photoprotective capacity was found in a provenance displaying the lowest stomatal response to drought, indicating a close interaction between different drought response mechanisms: In provenances with a rather anisohydric regulation of stomatal conductance, photosynthesis might be less impaired under drought and the photoprotective capacity might have lower priority in these provenances than in those with isohydric behaviour. The stomatal control under drought was shown by isotopic analyses in tree rings and leaf carbon pools, confirmed by instantaneous gas exchange measurements on the leaf. These concurrent results support the comparability of instantaneous and retrospective methods.

We studied six provenances belonging to the coastal variety, and three interior provenances. The regions of origin differed greatly in mean annual precipitation and temperature (362-2466 mm and 5.2-10.7 °C, respectively). Amongst coastal and interior provenances were some with extremely dry summer conditions in the region of origin. Our results show that different Douglas-fir provenances clearly varied in their drought sensitivity. Provenances originating from humid areas of the coastal zone showed high intrinsic water-use efficiency and carbon assimilation rates under average conditions as assessed by isotopic and gas exchange data, potentially explaining the high growth potential under average conditions. The rather isohydric regulation of stomatal conductance is linked to a medium to strong stomatal closure under drought, supported by enhanced instantaneous photoprotection. This mechanism led to strong growth decline under severe drought and might risk carbon starvation during prolonged drought. Thus, further studies on long-term drought effects are needed to evaluate the growth potential of these provenances under future climatic conditions.

Coastal provenances from regions with very dry summer conditions responded least to drought by stomatal closure and growth decline. In one of these provenances high drought resistance was combined with medium to high growth potential under past average conditions at Southwest German field sites, and should thus be investigated further. However, lowest assimilation rates and growth potential under average conditions were observed in the coastal provenance originating from the region with driest summers among all provenances studied here. This provenance showed an anisohydric regulation of stomatal conductance under dry growing conditions, supported by high levels of osmotic adjustment. High monoterpene emissions might contribute to the drought resistance. An interior provenance from arid origin showed highest assimilation rates under humid conditions, contradicting the low long term height growth. The high antioxidant, long term adjusted photoprotective, drought and heat protective potential indicate genotypic adaptation to dry and warm environments. We found mechanisms of drought resistance in provenances from dry regions of origin, both coastal and interior, which apparently impose carbon costs reflected in the low growth potential under moderate environmental conditions. Using provenances adapted to dry and warm environments might be decisive for forest management in regions where more arid summer conditions are projected for the future. Site-specific climate evaluations are thus needed to select the appropriate provenances.

Zusammenfassung

In Mitteleuropa werden zukünftig häufigere Trocken- und Hitzeperioden mit wirtschaftlichen Einbußen in der Waldwirtschaft erwartet. Die Douglasie (*Pseudotsuga menziesii* (Mirb.) Franco) wird oft als Alternative für die wirtschaftlich bedeutsame, jedoch trockenheitsempfindliche Fichte diskutiert (*Picea abies* (L.) H.Karst.). Zwei Unterarten, die Küsten- (FDC) und die Inlandsdouglasie (FDI), sind im ausgedehnten natürlichen Verbreitungsgebiet in Nordamerika beheimatet, welches ein großes Potenzial für die Auswahl produktiver und trockenresistenter Herkünfte bietet. Unser Ziel war, die Trockenreaktion verschiedener Douglasienherkünfte unter Verknüpfung morphologischer und physiologischer Parameter und die der Trockenheitsresistenz bzw. -empfindlichkeit zugrundeliegenden Mechanismen zu erforschen. Ein seit 1958 bestehender Herkunftsversuch in Südwestdeutschland ermöglichte die Untersuchung 50-jähriger Douglasien verschiedener Herkünfte entlang eines Höhengradienten. Unter kontrollierten Bedingungen simulierten wir die Effekte einer Hitzewelle auf Jungbäume zweier Provenienzen. Wir analysierten die Isotopenzusammensetzung ($\delta^{13}C$ und $\delta^{18}O$), den Gaswechsel der Blätter, Veränderungen im Stoffwechsel und das Baumwachstum.

Unsere Ergebnisse zeigen bei FDC aus humiden Regionen hohe Assimilationsraten (A_n) und ein starkes Wachstum unter moderaten Bedingungen. Unter Trockenheit wird die stark verringerte stomatäre Leitfähigkeit unterstützt durch Photoprotektion, allerdings unter großen Wachstumseinbußen. FDC aus Regionen mit starker Sommertrockenheit reagierten nur schwach mit Stomataschluss und Wachstumseinbußen auf Trockenheit, jedoch mit starker Osmoregulation und Monoterpen-Emissionen, welche zur Trockenresistenz beitragen könnten. Unter moderaten Bedingungen waren Wachstum und A_n teils gering. FDI aus einer ariden Region zeigten trotz hoher A_n nur ein geringes Wachstumspotenzial unter moderaten Bedingungen. Stark antioxidative und photoprotektive Mechanismen führen hier möglicherweise zu einer Kohlenstoffverteilung auf Kosten des Holzwachstums.

Die Herkünfte unterscheiden sich stark in ihrer Trockenreaktion und ihren Schutzmechanismen. Der Anbau trockenresistenter Herkünfte wird an Standorten von Vorteil sein, für die eine Häufung von ariden Sommerperioden vorhergesagt wird. Standortspezifische Klimabeurteilungen sind daher eine Voraussetzung für die passende Herkunftswahl und ein erfolgreiches Forstmanagement unter zukünftigen klimatischen Bedingungen.

1 Introduction

1.1 Impacts of climate change on forests

Elevated temperature, shifts in precipitation patterns and altered frequency of extreme events such as heat waves, prolonged drought or storms associated with global climate change (Easterling *et al.*, 2000; Meehl & Tebaldi, 2004; IPCC, 2013) are expected to affect forest growth, physiological performance and the distribution of species (IPCC, 2007). Drought is considered an important factor leading to increased forest dieback (Breshears & Cobb, 2005; Eilmann & Zweifel, 2011), and global climate change is likely to cause widespread forest decline in regions where drought events are predicted to increase in duration and severity (Allen *et al.*, 2010). However, current vegetation and earth systems models that include CO_2 fertilization and temperature benefits to growth, lack implementation of realistic mortality processes, leading to over-optimistic projections in tree growth (Allen *et al.*, 2015; Anderegg *et al.*, 2015b). Instead of growth benefits, a decrease in stomatal opening (leading to increases in intrinsic water-use efficiency) has been linked to increasing CO_2 levels (and at the same time enhanced evaporative demand) during the twentieth century as assessed by long-term $\delta^{13}C$ tree-ring measurements across European forests (Frank *et al.*, 2015). Moreover, recent significant increases in drought severity, linked to increasing temperatures as a major cause, have been already reported for the western USA (Peters *et al.*, 2015) and Central Europe (Briffa *et al.*, 2009). Thus, acclimation of forest trees to changing climatic conditions and their resistance, vulnerability, or resilience against extreme events will determine forest productivity and trees species in the future (Bréda *et al.*, 2006).

In fact, trees might be already responding to climate change as shown by increases in drought-induced tree mortality in many ecosystems (Allen et al., 2010). In some regions, tree ring analyses reveal that drought stress in the last 800-1200 years was most severe in recent years and was related to warming (Touchan *et al.*, 2011; Park Williams *et al.*, 2012; Griffin & Anchukaitis, 2014). Angiosperm trees in all forest biomes are already, under current conditions, operating at extremely narrow hydraulic safety margins (Choat et al., 2012), implying that a minor increase in drought intensity might induce levels of xylem embolism that will impair growth and lead to tree mortality. Gymnosperm trees are generally operating at wider

safety margins (Choat et al., 2012), suggesting that these trees may have a higher resistance to increased drought (Engelbrecht, 2012). Nevertheless, these trees might as well be threatened by hydraulic failure, as regional die-offs of pines show (McDowell *et al.*, 2008; Allen *et al.*, 2010). Furthermore, safety margins were shown largely independent of mean annual precipitation, explaining why drought-induced forest decline is occurring not only in arid regions but also in wet forests (Phillips et al., 2009; Meir & Woodward, 2010). These studies are first indications that forest diebacks will probably become more widespread, more frequent and more severe.

Norway spruce (*Picea abies* (L.) H.Karst.) is, economically, one of the most important tree species in Central Europe. However, this species is particularly threatened by drought (Mäkinen *et al.*, 2001; Gaul *et al.*, 2008; Ge *et al.*, 2010; Lebourgeois *et al.*, 2010) and consequently the economic implications are quite large (Hanewinkel *et al.*, 2013). Consequently, more drought tolerant species are discussed as potential replacement species.

In North America as well as in Europe, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is an economically important, remarkably productive, and relatively drought tolerant coniferous species (Brandl 1989; Heidingsfelder and Knoke 2004). Thus, identification of productive and drought-tolerant Douglas-fir provenances is expected to help mitigating the consequences of future climate change for forestry in Central Europe.

1.2 Mechanisms behind drought resistance

The current understanding of adaptation and acclimation potentials of trees to climatic change is rather incomplete. Hydraulic failure and carbon starvation under drought stress are two of the main causes for plant productivity loss and mortality (McDowell *et al.*, 2008; Anderegg *et al.*, 2015a; Sperry & Love, 2015 and many others), interlinked with nutrient availability (e.g. Ewers *et al.*, 2000; Dziedek *et al.*, 2016; Gessler *et al.*, 2016; Meyer-Grünefeldt *et al.*, 2016) and biotic attacks (Fangmeier *et al.*, 1994; McDowell, 2011; Hartmann *et al.*, 2013; Hentschel *et al.*, 2014; Anderegg *et al.*, 2015a). Trees have evolved adaptations to drought stress (see Figure 1) such as drought induced changes in leaf physiology and wood anatomy (Rossi *et al.*, 2013), photoprotective mechanisms in the leaves (Baroli & Niyogi, 2000; Munekage et al., 2002; Peñuelas & Munné-Bosch, 2005) or osmotic adjustment (Bohnert *et al.*, 1995; Hasegawa *et al.*, 2000; Chen *et al.*, 2007; Cuin *et al.*, 2010). Xylem resistance to embolism,

one of the key adaptive mechanisms in trees (Choat *et al.*, 2012; Lens *et al.*, 2013), has been linked to wood lignin content (Voelker *et al.*, 2011), wood density fluctuations (Hacke *et al.*, 2001; Jacobsen *et al.*, 2007; Battipaglia *et al.*, 2014), and wood morphological traits (Baas, 1976; Jansen *et al.*, 2012). Cavitation resistant wood contributes to the comparably high drought resistance in Douglas-fir (Anekonda et al., 2002; Warren et al., 2003) and wood density was shown to be involved in adaptation to drought in Douglas-fir (Dalla-Salda *et al.*, 2009, 2011). High proportions of cavitation-resistant dense wood were shown to increase the survival of Douglas-fir under severe drought (Martinez-Meier *et al.*, 2008). However, the investment into cavitation-resistant dense wood could also contribute to growth limitations (Jyske *et al.*, 2010).

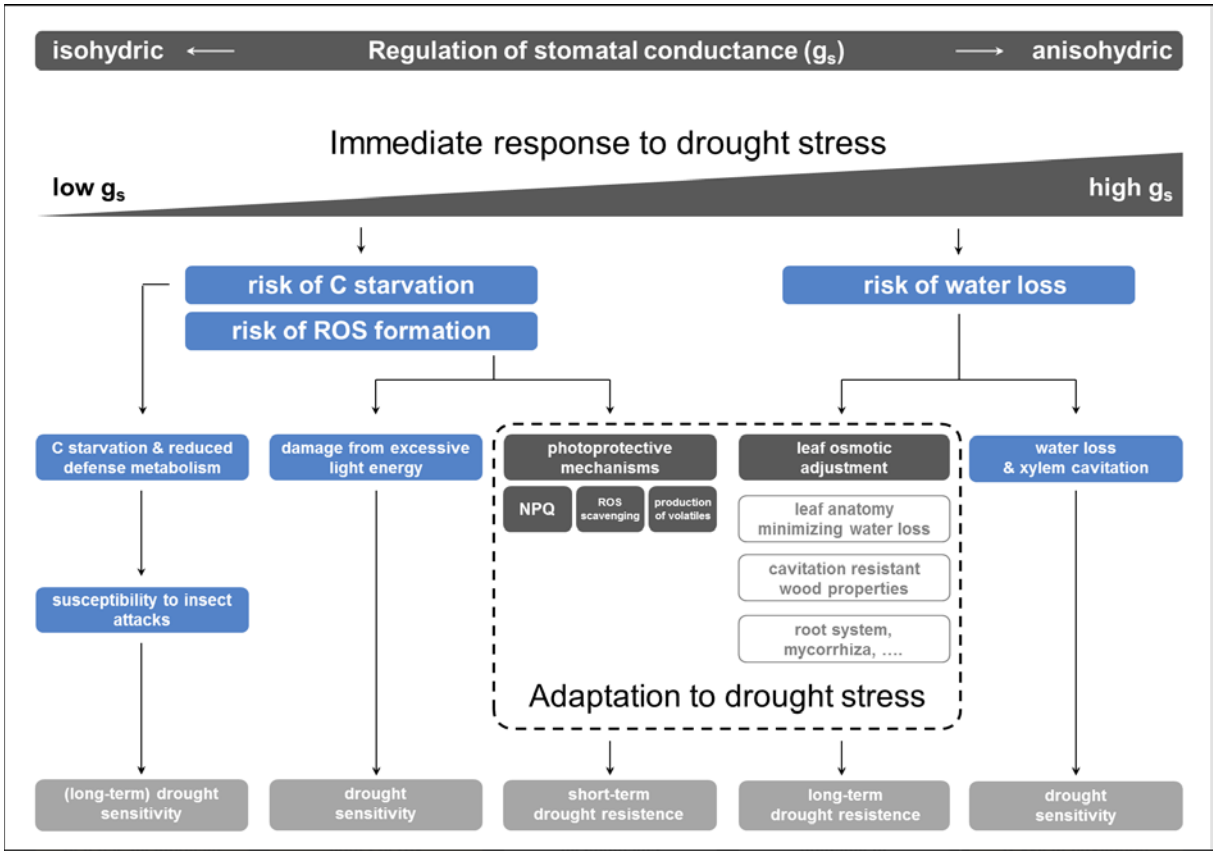

Figure 1 Mechanisms behind drought sensitivity and resistance, and their association with water loss, carbon (C) starvation, and formation of reactive oxygen species (ROS)
The figure shows examples for potential stress response mechanisms of the plants (dark grey for mechanisms discussed in this thesis, and white for other mechanisms), risks and damages (blue) and the resulting resistance or sensitivity (light grey)

Physiological adaptation to drought stress includes the regulation of stomatal conductance (g_s), minimizing water loss through transpiration but at the same time reducing CO_2 supply for photosynthesis (Chaves, 1991; Cornic & Massacci, 1996; Sevanto *et al.*, 2014) leading to declines in carbon assimilation (A_n) and growth. A strong stomatal response to drought is part of the isohydric regulation of g_s (avoidance of physiological drought), maintaining plant water status at the cost of reduced carbon assimilation (Hubbard *et al.*, 2001; Mcculloh *et al.*, 2014), risking carbon starvation (McDowell *et al.*, 2008). In contrast, anisohydric plants maintain relatively high g_s and A_n under drought but potentially being more strongly exposed to hydraulic failure (Sade *et al.*, 2012; Mitchell *et al.*, 2013; Sevanto, 2014).

g_s and A_n can be assessed by instantaneous leaf gas exchange measurements and are used to derive the intrinsic water-use efficiency (IWUE) as the ratio of A_n to g_s. The effects of changes in g_s and A_n on the leaf internal CO_2 concentration (c_i) are additionally imprinted onto the carbon isotope composition ($\delta^{13}C$) of recent assimilates (Farquhar *et al.*, 1982; Brugnoli *et al.*, 1988; Cernusak *et al.*, 2005). This is because $\delta^{13}C$ is besides its dependence on the isotopic composition of CO_2 affected by the photosynthetic carbon isotope fractionation in plants that in turn is determined by fractionation during CO_2 diffusion into the leaf, associated with CO_2 dissolution and of enzyme reactions during assimilation. Due to its dependence on g_s and A_n, $\delta^{13}C$ is directly related to IWUE (Farquhar *et al.*, 1989). $\delta^{13}C$ can be analysed in different plant tissues and fractions to derive physiological information integrating over varying time spans (see chapter 5.1).

Furthermore, stomatal and photosynthetic effects on $\delta^{13}C$ can be disentangled using a dual isotope approach based on carbon and oxygen isotope composition in plant organic matter (Scheidegger *et al.*, 2000; Barbour *et al.*, 2002; Ripullone *et al.*, 2009; Gessler *et al.*, 2009a; Barnard *et al.*, 2012). $\delta^{18}O$ of recent assimilates depends on the one hand on the isotopic composition of the source water. On the other hand it is determined by the evaporative ^{18}O enrichment of the leaf water. This enrichment depends directly on the water vapour pressure (VPD) difference between the leaf interior and the atmosphere (Dongmann *et al.*, 1974). Since stomatal conductance is also mainly driven by that VPD difference, there is an indirect negative relationship between g_s and evaporative enrichment. Moreover the Péclet effect, i.e. the dilution of evaporatively enriched water in the leaf lamina by non-enriched xylem water (Farquhar & Lloyd, 1993) is directly related to transpiration and thus under stomatal control

(Gessler et al. 2009). As a consequence of two effects, the evaporative enrichment of leaf water is indicative of g_s. The leaf water isotopic composition is imprinted on the assimilates during the isotopic exchange between carbonyl oxygen atoms of organic molecules and the local cell water (Barbour, 2007). Since $\delta^{18}O$ of plant organic matter is only related to g_s, but not to variations in photosynthetic capacity (A_{max}) (Barbour *et al.*, 2000a; Scheidegger *et al.*, 2000), combined measurements of $\delta^{13}C$ and $\delta^{18}O$ allow a separation between stomatal and photosynthetic effects on $\delta^{13}C$ (Scheidegger *et al.*, 2000; Barbour *et al.*, 2002; Ripullone *et al.*, 2009; Gessler *et al.*, 2009a; Barnard *et al.*, 2012).

Douglas-fir displays a rather anisohydric regulation of stomatal conductance in combination with cavitation resistant wood (Anekonda et al., 2002; Warren et al., 2003) and leaf anatomical and biochemical traits associated with leaf longevity and drought resistance (Warren & Adams, 2004). Regarding biochemical traits, osmotic adjustment in the leaves can increase the resistance towards environmental drought by regulating plant cell turgor via synthesis of organic osmolytes such as glycine betaine, proline and polyols (Bohnert et al., 1995; Hasegawa et al., 2000) or via accumulation of inorganic ions such as Na^+, Cl^- and K^+ (Chen et al., 2007; Cuin et al., 2010; Shabala & Shabala, 2011).

Stomatal closure decreases the leaf internal CO_2 concentration and thus impairs photosynthetic CO_2 fixation in C3 plants. Consequently, light energy cannot be quenched photochemically and might support the formation of reactive oxygen species (ROS; Niyogi, 2000; Ensminger *et al.*, 2006). Photoprotective mechanisms in plants involve non-photochemical quenching, scavenging of ROS and production of volatiles (Baroli & Niyogi, 2000; Munekage et al., 2002; Peñuelas & Munné-Bosch, 2005).

The studies included in this thesis comprise analyses of tree responses towards varying environmental conditions. I assessed effects on growth and responses on the physiological and metabolic level which are interlinked with each other. The analyses stretching from biochemistry to growth were conducted in order to (1) identify productive and drought-tolerant Douglas-fir provenances and (2) to understand mechanisms of drought resistance or sensitivity.

1.3 The natural range of Douglas-fir and the benefit of using provenance trials

Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) grows within an largely extended natural range in Northwest America (see Figure 2; Campbell, 1991; Dean, 2007; Gugger *et al.*, 2010). The evolutionary history of Douglas-fir is shaped by the rise of the Cascade Range and Sierra Nevada during the Pliocene, imposing a rain shadow in the Columbia Plateau and Great Basin (Brunsfeld *et al.*, 2000), and thus explaining the separation of Douglas-fir populations into two varieties, the coastal (var. *menziesii*) and the interior (var. *glauca*) Douglas-fir, as also indicated by fossil records. Further population divergence within each variety took place during Pleistocene glacial cycles (Gugger & Sugita, 2010).

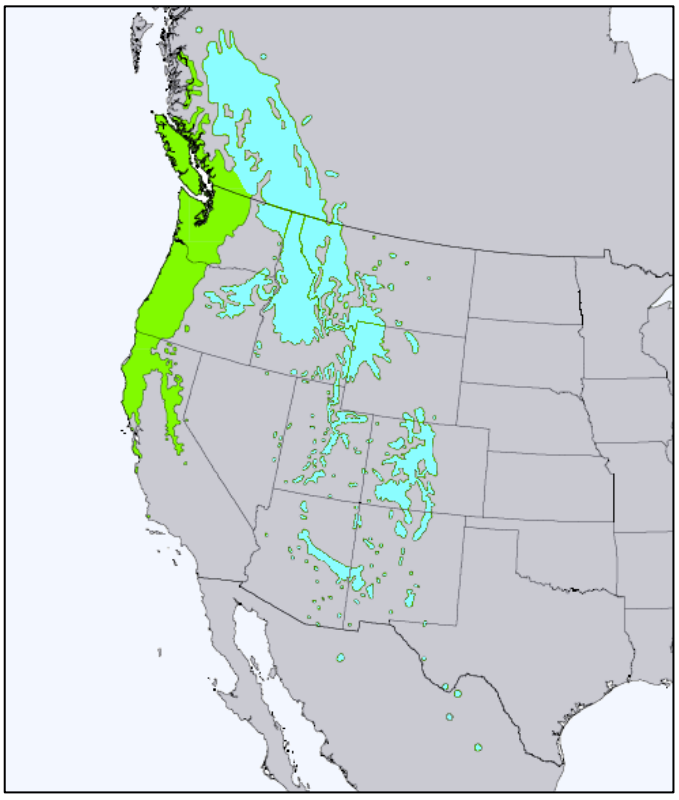

Figure 2 Modern range of the coastal (green) and the interior (blue) variety of Douglas-fir
Figure modified from Little (1999)

Postglacial recolonization of Canada by both varieties resulted finally in the *menziesii-glauca* transition zone which is mainly located in Southern Interior British Columbia (Little, 1971; Sorensen, 1979; Gugger *et al.*, 2010). Today, the natural range of the coastal variety of Douglas-fir extends from north to south over 2,000 km from the Pacific Northwest's fog-belt to the

dry coastlands of southern California. The interior variety extends over 4,500 km along the dry continental climates of the montane to the subalpine Rocky Mountains from Alberta to Colorado. The species grows from sea level up to elevations of 3,000 m asl (Hermann & Lavender, 1999). Through isolation, redistribution and adaptation to contrasting ecozones over a vast area of distribution, Douglas-fir has evolved a large variety of genetically diverse populations (Campbell, 1991; Dean, 2007). Douglas-fir provenance trials thus provide an excellent basis to compare the growth performance and drought resistance of different provenances under similar site conditions (St Clair *et al.*, 2005; Eilmann *et al.*, 2013).

Interior and coastal provenances show genetic (Krutovsky & Neale, 2005; Müller *et al.*, 2014), phenotypic (Krutovsky & Neale, 2005; St Clair *et al.*, 2005; Eilmann *et al.*, 2013; Sergent *et al.*, 2014) and physiological differences (Bansal *et al.*, 2014, 2016). Genes involved in drought resistance showed a high genetic differentiation between interior and coastal Douglas-fir suggesting a different evolution of the two varieties (Müller *et al.*, 2014). Current studies are aiming at identifying provenances with a high growth potential and drought resistance but the resulting recommendations for future forest management are diverse: In North America, mixtures of local seed sources with seed sources from lower elevations and farther south have been recommended to ensure productive and adapted Douglas-fir forests in the face of climate change (Ledig & Kitzmiller, 1992; Bradley St Clair & Howe, 2007). However, studies on water relations in Douglas-fir seedlings suggested that populations from regions with relatively cool winters and arid summers are potentially best adapted to warm and dry conditions (Bansal *et al.*, 2014). Considering the growth performance of provenances during past decades, Washington provenances from humid coastal areas and from relatively drier inland areas were shown top performers during the period 1961-1990 across Western Europe both by studying the growth potential in provenance trials and by applying bioclimate envelope models to climate scenarios for Western Europe for the same time period (Isaac-Renton *et al.*, 2014). Nevertheless, provenances that have proven good productivity at European sites under past environmental conditions might not be adapted to climatic conditions in the future. In fact, Isaac-Renton *et al.* (2014) demonstrated that climate observations over the recent three decades justify modification of the current usage of Douglas-fir provenances in forest plantations in western and central Europe. Therefore, present prove-

nance studies should aim at selecting provenances suitable to grow and persist under the anticipated rapidly changing future climatic conditions.

1.4 Aims and studies

The research presented in this thesis is part of an interdisciplinary project funded by the German Research Foundation 'Adaptation of Forest Trees to Climatic Change - Diversity of Drought Responses in Douglas-Fir Provenances (*Pseudotsuga menziesii* (Mirb.) Franco)'. The aim of the joint research program is to study the drought response diversity of various Douglas-fir provenances from the molecular to the organism level, and to link phenotypic and physiological variation (as expressed in e.g. growth responses and isotope composition) with allelic variation in candidate genes. Furthermore, metabolite profiles as physiological markers were performed with external funds and linked to the results of the joint project. The focus of this thesis lies on linking the information derived from leaf gas exchange (assessed by leaf isotopic analyses and gas exchange measurements) to metabolic changes that interact with leaf gas exchange (for instance osmotic adjustment) as well as to concomitant changes in growth patterns under heat and drought stress.

The thesis consists of field experiments (chapters 2 and 3) and experiments under controlled conditions (chapter 4). In joint field campaigns, the interdisciplinary project offered the unique opportunity to study 50 year old Douglas-fir trees (ca. 25 - 35 m tall) in a provenance trial (Table 1) monitored by the Forest Research Institute Baden-Württemberg (Freiburg, Germany) since they were established (Kenk & Thren, 1984).

Table 1 Location and climatic conditions at the field sites of the provenance trial (Kenk & Ehring 2004)

Field Site	Region	Altitude (m asl)	MAT (°C)	MAP (mm)	See field experiment in chapter
Dgl 122 Wiesloch	Rhine valley	105	9.9	660	2
Dgl 115 Mooswald	Rhine valley	228	10.0	830	3
Dgl 114 Illenberg	Black Forest	500	8.0	903	3
Dgl 116 Schauinsland	Black Forest	940	6.0	1791	3
Dgl 123 Schluchsee	Black Forest	1050	6.1	1345	2

Table 2 Geographic location and climatic conditions in the regions of origin of the provenances studied in the field experiments (chapter 2 & 3) and experiments under controlled conditions (chapter 4)
Longitude and latitude are given in decimal format. Mean annual temperature (MAT), mean annual precipitation (MAP), mean summer temperature (May-Sep, MST), mean summer (May to Sept.) precipitation (MSP), annual heat:moisture index (AHM=(MAT+10)/(MAP/1000)), summer heat:moisture index (SHM=mean warmest month temperature/(MSP/1000)), Hargreaves climatic moisture deficit (CMD) were modelled based on ClimateWNA v4.72 (© University of British Columbia, Wang *et al.*, 2012). Elevation according to Kenk & Ehring (2004) or according to information given by the seedling supplier (Monte Creek and Pend Oreille). Physiographic regions in USA according to Franklin & Dyrness (1973)

Provenance name *Region (Province / State)*	Variety	Elevation (m)	North latitude	West longitude	MAT (°C)	MAP (mm)	MST (°C)	MSP (mm)	AHM	SHM	CMD	See chapter		
Salmon Arm (31/102) *Southern Interior (BC, Canada)*	Interior	580	50.22	119.22	6.2	415	14.6	188	39	93.9	426	2		
Monte Creek *Southern Interior (BC, Canada)*	Interior	850	50.65	119.96	5.2	362	13.6	171	42	96.9	433			4
Cameron Lake *Vancouver Island, East Coast (BC, Canada)*	Coastal	210	49.25	124.67	7.7	2295	13.3	307	7.7	51.4	167	2	3	
Duncan Paldi *Vancouver Island, East Coast (BC, Canada)*	Coastal	260	48.75	123.83	8.3	1114	13.8	170	16.5	94.8	292		3	
Conrad Creek (Darrington 3) *North Cascades, West side (WA, USA)*	Coastal	280	48.25	121.50	10.7	2390	17.1	415	8.7	46.9	184	2	3	
Pend Oreille *Okanogan Highlands (WA, USA)*	Interior	925	48.28	117.73	6.5	736	14.8	220	25.4	93.2	397			4
Timber *Coast Range (OR, USA)*	Coastal	270	45.80	123.38	9.7	2046	15.0	233	9.6	74.8	315		3	
Santiam River *West Cascades (OR, USA)*	Coastal	800	44.67	121.97	8.8	1801	14.9	271	10.5	64.8	346	2	3	
Pamelia Creek *West Cascades (OR, USA)*	Coastal	750	44.66	121.83	10	2466	15.1	430	8.1	40.8	185		3	

We studied provenances from British Columbia, Washington and Oregon including the coastal and interior variety of Douglas-Fir, originating from environments differing strongly in temperature (Table 2), precipitation amount (Figure 3) and, consequently, climatic moisture deficit (CMD, Table 3).

Figure 3 Monthly precipitation in the regions of origin of the provenances studied in the field experiments and in the experiments under controlled conditions
Precipitation was modelled with ClimateWNA v4.72 (© University of British Columbia, Wang *et al.*, 2012)

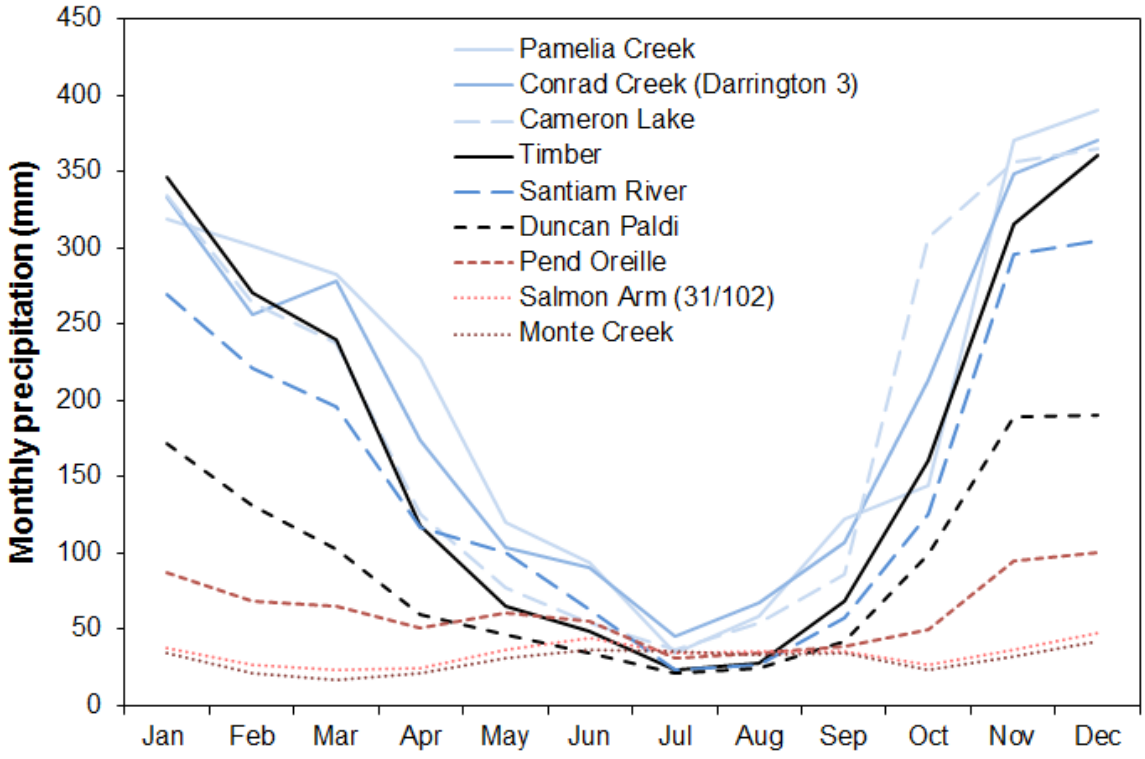

Table 3 Monthly Hargreaves climatic moisture deficit (CMD) in the regions of origin of the provenances studied in the field experiments and experiments under controlled conditions
CMD was modelled with ClimateWNA (© University of British Columbia) v4.72 (Wang *et al.*, 2012)

Provenance name	CMD Jan	CMD Feb	CMD Mar	CMD Apr	CMD May	CMD Jun	CMD Jul	CMD Aug	CMD Sep	CMD Oct	CMD Nov	CMD Dec
Salmon Arm (31/102)	0	0	11	42	66	81	106	82	33	5	0	0
Monte Creek	0	0	17	43	68	84	101	81	31	8	0	0
Cameron Lake	0	0	0	0	8	43	75	41	0	0	0	0
Duncan Paldi	0	0	0	0	40	66	92	72	22	0	0	0
Conrad Creek (Darrington 3)	0	0	0	0	5	33	93	53	0	0	0	0
Pend Oreille	0	0	1	21	45	72	120	95	41	3	0	0
Timber	0	0	0	0	34	66	112	91	12	0	0	0
Santiam River	0	0	0	0	7	68	130	108	33	0	0	0
Pamelia Creek	0	0	0	0	0	25	99	61	0	0	0	0

The main part of this thesis includes three studies on growth, physiological and metabolic responses towards environmental conditions (chapter 2, 3, and 4). The appendix includes two studies from the joint project that are closely interlinked with the aforementioned studies (Du *et al.*, 2014; Junker *et al.*, 2017).

For studies on the response of Douglas fir to environmental drivers during two growing seasons, we selected two sites from the provenance trial representing the end members of an altitudinal transect and thus showing contrasting temperature and precipitation regimes (Dgl 122 Wiesloch, 105 m asl in the Rhine valley and Dgl 123 Schluchsee, 1050 m asl in the Black-Forest, see Table 1), and 4 provenances originating from climatically contrasting environments (see Table 2). Joint field campaigns conducted in the growing seasons 2010 and 2011 led to two studies presented in chapter 2 and in the appendix, (Junker *et al.*, 2017).

For retrospective analyses of the response of Douglas fir to environmental drivers, including the hot and dry year 2003, we studied 6 provenances (Table 2) along an altitudinal transect at three sites close to Freiburg (230, 500 and 940 m asl, Table 1). The results are shown in chapter 3 (Jansen *et al.*, 2013).

In an experiment under controlled conditions, we exposed 3- and 4-year old Douglas-fir seedlings of two provenances (Table 2) to elevated temperature and atmospheric drought and assessed the physiological and metabolic responses to understand the mechanisms of drought resistance and susceptibility. This experiment lead to two studies, presented in chapter 4 (Jansen *et al.*, 2014) and in the appendix (Du *et al.*, 2014).

In order to identify productive as well as heat and drought-tolerant Douglas-fir provenances, the studies included in this thesis aim at detecting physiological and metabolic markers for drought and heat sensitivity or resistance of provenances and linking the physiological variation to phenotypic (i.e. growth) variation. Stable isotope techniques and leaf gas exchange were applied to characterise intrinsic water-use efficiency and stomatal reactions. Changes in the metabolite profiles were analysed to detect marker metabolites for heat stress responses and to identify changes in metabolic pathways in reaction to heat. Leaf levels of organic osmolytes and inorganic ions were studied to reveal information on osmotic adjustment in response to heat and drought. These results are complemented by the study on photoprotective mechanisms (see appendix).

On different time-integrating scales and under field and controlled conditions, these studies overall intend to answer the following research questions:

Do Douglas-fir provenances differ in physiological, metabolic and growth parameters under humid as well as hot and dry conditions?

Can we derive information on heat and drought sensitivity and resistance regarding short-term and long-term responses?

Can we elucidate stress response mechanisms linking physiological and morphological parameters such as stomatal control (chapter 2 & 3), growth responses (chapter 3), osmotic adjustment (chapter 2 & 4), changes in metabolic pathways (chapter 4 & appendix) or photoprotective mechanisms (appendix)?

Do the environmental conditions at the site of origin play a role in this response and if so, what are specific results for provenances originating from warm and dry areas versus humid areas?

2 Leaf Stable Isotope Composition, Gas Exchange and Osmotic Adjustment in Douglas-fir

Title: Site-Specific Climate Sensitivity: Relations between Leaf Stable Isotope Composition, Gas Exchange and Osmotic Adjustment in Douglas-fir

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2.1 Abstract

Plant growth and functioning can be severely affected by drought and high temperature. Leaf stomatal responses and osmotic adjustment have both an impact on leaf water content, gas exchange, and thus growth, but their interplay highly depends on plant type and environmental conditions. We applied different methodological approaches based on integrative measures, such as the analysis of leaf carbon and oxygen isotopic composition ($\delta^{13}C$ and $\delta^{18}O$) and mineral content, as well as instantaneous leaf gas exchange measurements to assess the physiological response of fifty-year-old Douglas-fir trees of four provenances (*Pseudotsuga menziesii* (Mirb.) Franco) to varying environmental conditions at two contrasting field sites.

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At the humid site, leaf water content was positively, and leaf $\delta^{13}C$ negatively correlated with water availability. The relationship between leaf $\delta^{13}C$ and gas exchange parameters shows a dominant control of $\delta^{13}C$ through stomatal conductance. This was not observed at the dry site. $\delta^{13}C$ was also not correlated to air humidity and soil water content, whereas leaf mineral and water content were negatively correlated with water availability. These results suggest that trees growing at the dry site controlled their osmotic potential to maintain leaf water content keeping stomatal conductance rather constant.

When assessing the physiological response of trees through stable isotope analyses and photosynthetic gas exchange measurements we need to take into account the effect of osmotic regulation on leaf physiology, which might differ among sites and according to the long-term acclimation of trees.

2.2 Introduction

Photosynthetic CO_2 uptake by plants is inevitably coupled to the loss of water vapour from leaves. As a consequence, drought and high temperature directly affect the physiology of plants and can lead to growth cessation, predisposal to diseases, severe damage and mortality (Allen et al., 2010). An instantaneous plant response to avoid excessive water loss is the regulation of stomatal conductance (Chaves, 1991; Cornic & Massacci, 1996), which at the same time limits CO_2 uptake through stomata and diffusion to the chloroplasts. Another adaptive trait towards heat and drought is osmotic adjustment (OA), enabling an adjustment of plant cell turgor and, thus, possibly interacting with leaf gas exchange (GE). This interplay between OA and GE under drought might affect the reliability of single parameters as markers for drought sensitivity.

Gas exchange-related parameters can be assessed in different ways: Net assimilation rate (A_n) and stomatal conductance (g_s) can be assessed instantaneously with GE measurements on the leaf. The ratio of carbon gain to water loss, i.e. A_n to g_s, is described by the intrinsic water-use efficiency ($IWUE_{GE} = A_n / g_s$), a main driver in the global cycles of water and carbon (Drake et al., 1997; Keenan et al., 2013). Leaf physiological changes are also imprinted onto the carbon and oxygen isotopic composition ($\delta^{13}C$ and $\delta^{18}O$) of recent assimilates (Farquhar et al.,

1982; Brugnoli et al., 1988; Cernusak et al., 2005). $\Delta^{13}C$ is in a first approximation proportional to the intercellular CO_2 concentration (c_i) (and more accurately to the chloroplastic concentration, cc) and consequently depends on both maximum photosynthetic capacity (A_{max}) and g_s. As a consequence, $\Delta^{13}C$ and $\delta^{13}C$ are widely used as indicators of $IWUE_{13C}$ (Farquhar et al., 1989).

$\delta^{18}O$ of plant organic matter is, via VPD and the evaporative enrichment of leaf water (Sternberg & DeNiro, 1983; Cernusak et al., 2005), as well as via the Péclet effect (Farquhar & Lloyd, 1993; Gessler et al., 2009b), related to g_s, but not to variations in A_{max} (Barbour *et al.*, 2000a; Scheidegger *et al.*, 2000). In contrast, $\delta^{13}C$ is related to both (Farquhar et al., 1989), so that combined analyses of $\delta^{13}C$ and $\delta^{18}O$ allow for a separation between stomatal and photosynthetic effects on $\delta^{13}C$ (Scheidegger et al., 2000; Barbour et al., 2002; Ripullone et al., 2009; Gessler et al., 2009a; Barnard et al., 2012).

The isotopic composition of different plant tissues and organic matter fractions can be used to infer physiological information that integrates plant responses to the environment over different time spans (e.g. Fotelli et al., 2003). The water-soluble organic matter (WSOM) of leaves can be representative of recent assimilates integrating IWUE signals over hours up to several days (Gessler et al., 2009c). The isotopic signature is further transferred to the total organic matter (TOM) of leaves in an ongoing process over the growing season. TOM isotopic composition is considered a parameter that contains a strong isotopic signal from the time the leaf was produced, but, due to the turnover of structural and mainly non-structural compounds, also integrates physiological signals over several weeks or months up to the whole growing season or the lifespan of the leaf (c.f. Fotelli et al., 2003).

Via a coordinated control of multiple cellular processes, stomata open or close in response to several environmental stimuli (e.g. Hetherington & Woodward, 2003; Shimazaki et al., 2007). It has been suggested that stomatal conductance is affected by plant cell turgor and leaf water content (WC, Comstock & Mencuccini, 1998), as well as xylem pressure within the leaf rachis (Cochard et al., 2002). In the acclimation of plants to heat and drought, osmotic regulation plays a crucial role and adjusts plant cell turgor by synthesis of organic osmolytes, and by controlling inorganic ion fluxes through cellular membranes. Thus osmotic regulation can, indirectly, influence stomatal aperture and consequently $\delta^{13}C$ and $\delta^{18}O$ of plant organic matter. Organic osmolytes such as glycine betaine, proline and polyols are often accumulated

under drought or salinity (Bohnert et al., 1995; Hasegawa et al., 2000). The biosynthesis of organic osmolytes is, however, metabolically expensive and a relatively slow process, whereas OA by accumulation of inorganic ions, mainly Na^+, Cl^- and K^+, is an alternative, contributing to plant osmoregulation (Chen et al., 2007; Cuin et al., 2010; Shabala & Shabala, 2011).

Leaf stomatal responses and OA both sustain WC and leaf functioning under drought, but their interplay might depend on the plant species or even provenance, and on growing conditions. We therefore conducted experiments in the canopy of 50 year-old Douglas-fir trees at two contrasting field sites in south-western Germany, covering an elevation gradient of 945 m and a temperature and precipitation range of 3.8 °C and 685 mm, respectively, and with four genetically differentiated (Müller et al., 2015; Neophytou et al., 2016) Douglas-fir provenances at each site. Cameron Lake and Conrad Creek originate from humid areas in British Columbia and Washington State, whereas Santiam River is from a dry area in Oregon and Salmon Arm is from a very dry habitat in Southern Interior British Columbia.

We assessed the short term acclimation of leaf carbon and water dynamics comparing the IWUE derived from $\delta^{13}C$ in WSOM of needles ($IWUE_{13C}$), which describes a time integrated response over hours to days, with A_n, g_s and $IWUE_{GE}$ derived from instantaneous GE measurements of the leaf.

Furthermore, we related these short-term measures to the isotopic composition in leaf TOM that integrates over a longer term (i.e. up to the life span of the leaf). We applied a dual isotope approach ($\delta^{13}C$ and $\delta^{18}O$), following Scheidegger et al. (2000), to distinguish between the effect of A_{max} or g_s on $\delta^{13}C$. In order to study which environmental factors have impacts on the carbon isotope composition, we conducted correlation analyses over various time spans with weather and isotopic data ($\delta^{13}C_{WSOM}$ and $\delta^{13}C_{TOM}$). Finally, we analysed WC as well as the leaf mineral and potassium content (MC, K) as a proxy for OA.

Our hypotheses were: (1) The physiological information derived from different methods (GE and isotopic data) is closely related to each other.

(2) The leaf isotopic composition in Douglas-fir is correlated to environmental conditions at the field sites, with (2a) leaf WSOM isotopic composition being related to environmental conditions over a short period before sampling, (2b) leaf TOM isotopic composition being mainly related to the environmental conditions during the summer period, and (2c) prove-

nances from more humid regions of origin showing a larger sensitivity in their leaf isotopic composition towards environmental conditions.

(3) The leaf mineral content, as an indicator for osmotic regulation, is increased under dry conditions and alters leaf water content potentially affecting stomatal conductance, and thus $\delta^{13}C$ of plant organic matter. (3a) Osmotic adjustment is most pronounced in provenances originating from dry habitats.

2.3 Materials and Methods

Plant material and field sites

We examined 50 year-old Douglas-fir trees at two field sites in south-western Germany which are part of an international Douglas-fir provenance trial established in 1958. The sites Schluchsee and Wiesloch cover an elevation gradient of 945 m and differ substantially in climatic conditions and soil properties (Kenk & Ehring, 2004) leading to large differences in soil water availability (Table 4 & 5). Schluchsee, located in the southern Black Forest at an elevation of 1050 m asl, is a humid site with loamy soil, while Wiesloch represents the rather warm and dry climate of the Rhine valley at 105 m elevation asl with sandy soils. At Wiesloch, mean annual precipitation is by 685 mm lower and mean annual temperature is by 3.8 °C higher compared to the high elevation site Schluchsee.

We studied two coastal provenances from humid areas in British Columbia and Washington State (Cameron Lake and Conrad Creek, respectively, see Table 6), one coastal provenance from a relatively dry area in Oregon (Santiam River), and one Douglas-fir provenance from a very dry habitat in the *menziesii-glauca* transition zone in Southern Interior British Columbia (Salmon Arm). Genetic data (Müller et al., 2015; Neophytou et al., 2016) strongly indicate that Salmon Arm belongs to the interior variety.

Table 4 Location and climatic conditions at the field sites Schluchsee and Wiesloch (Kenk & Ehring 2004)

Field Site	Region	Location	Altitude (m asl)	MAT (°C)	MAP (mm)
Dgl 123 Schluchsee	Black Forest	N47°50'33", E8°6'54"	1050	6.1	1345
Dgl 122 Wiesloch	Rhine valley	N49°16'40", E8°34'35"	105	9.9	660

Table 5 Average weather conditions in the vegetation periods 2010 and 2011 at the field sites Schluchsee and Wiesloch
Average Soil water availability (compared to the site specific maximum) was modelled using the forest hydrological water budget model WBS3 (Keitel et al., 2006)

Field Site	Temperature (°C)		Sunshine (hrs)		Precipitation sum (mm)		Relative humidity (%)		Soil water availability (%)	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Dgl 123 Schluchsee	11.96	13.14	6.13	7.52	753.4	470.9	72.18	67.41	91	69
Year-to-year		+9.9%		+22.7%		-37.5%		-6.6%		-24.2%
Dgl 122 Wiesloch	16.91	17.91	6.96	6.98	410.2	278.8	64.92	64.84	39	22
Year-to-year		+5.9%		+0.3%		-32.0%		-0.1%		-43.6%

Table 6 Geographical position and climatic conditions in the regions of origin of the four provenances studied at Schluchsee and Wiesloch
Mean annual temperature (MAT), mean annual precipitation (MAP), mean summer temperature (May-Sep, MST), mean summer (May to Sept.) precipitation (MSP), annual heat:moisture index (AHM=(MAT+10)/(MAP/1000)), summer heat:moisture index (SHM=mean warmest month temperature/(MSP/1000)), Hargreaves climatic moisture deficit (CMD) were modelled based on ClimateWNA v4.72 (Wang et al. 2012). Elevation according to Kenk & Ehring (2004)

Provenance name Region (Province / State)	Elevation (m)	West longitude	North latitude	MAT (°C)	MAP (mm)	MST (°C)	MSP (mm)	AHM	SHM	CMD
Salmon Arm Southern Interior (BC)	580	119	50	6.2	415	14.62	188	39	93.9	427
Cameron Lake Vancouver Island, East Coast (BC)	210	124	49	7.7	2295	13.30	307	7.7	51.4	167
Conrad Creek North Cascades, West side (WA)	280	121	48	10.7	2390	17.08	415	8.7	46.9	183
Santiam River West Cascades (OR)	800	122	44	8.8	1801	14.92	271	10.5	64.8	346

Climate data for the region of origin as shown in Table 6 was obtained with the software ClimateWNA v.4.72 for the reference period (1961-1990) for specific locations based on latitude, longitude and elevation as described in Wang et al. (2012).

Meteorological data and soil water availability for the field sites

Meteorological data for the years 2010 and 2011 were obtained from weather stations in close proximity to the field sites Schluchsee (private meteorological station located 6 km from the site, N47°49’16”, E8°11’08”, 992 m asl) and Wiesloch (German Weather Service, DWD weather station in Waghäusel-Kirrlach 4 km from the site, N49°15’0”, E8°32’24”, 105 m asl). Average weather conditions of the vegetation periods 2010 and 2011 are presented in Table 5.

Sunshine hours (SH) are given as a proxy for radiation. Monthly weather data and seasonal patterns are presented in Junker et al. (2017). In May 2010, temperatures were exceptionally low at Schluchsee, leading to a delayed start of the growing season. In Schluchsee, the 2011 growing season precipitation was lower (-282.5 mm) compared to 2010. The 2011 growing season was also drier at Wiesloch compared to 2010 (-131.4 mm).

Field site specific precipitation and soil properties lead to large differences in soil water availability. We used the forest hydrological water budget model WBS3 to estimate daily total available soil water (TAW) as a percentage of maximum soil water availability based on the variables temperature, precipitation, latitude, soil type, plant cover, slope and slope aspect (Keitel et al., 2006; Junker et al., 2017). In terms of TAW, the driest period at Schluchsee was May 2011. Also, June 2011 and the beginning of July 2011 were relatively dry at Schluchsee with precipitation being below the long-term average (1961-1990). The driest period at Wiesloch was July 2010, followed by May 2011 and July 2011.

Field campaigns

Full measurement campaigns (including GE measurements and needle harvest) took place in May and July in the years 2010 and 2011 on both field sites. During each campaign, GE was measured on previous-year needles in the sunlit part of the canopies (n=5-6 per provenance) over a period of 2 weeks. GE measurements took place at heights between 24 to 29 meters using a hydraulic lift. Previous-year needles for isotope analysis were sampled at noon on a day at the end of the campaign. Furthermore, needle material was harvested in additional campaigns carried out in June and September of both years 2010 and 2011. Needle material of the sun-exposed crown of N=5-6 trees per provenance was sampled using shotguns or slingshots, removed from the twigs, immediately frozen in liquid nitrogen and stored at -80 °C.

At the high elevation site Schluchsee, growing season starts later than at Wiesloch. We conducted each campaign at Schluchsee after finishing the campaign at Wiesloch to account for the delay in phenology. Phenology of bud development was assessed at both sites (Bailey & Harrington, 2006; Junker et al., 2017).

Photosynthesis measurements

GE was measured using a LI-COR 6400 XT (LI-COR Biosciences, Lincoln, NE, USA) on previous-year needles within the sun-exposed part of the crown. Measurement conditions in the closed cuvette were set to 500, 1000 and 1500 µmol photons m^{-2} s^{-1} light intensity, 400 ml min^{-1} flow rate, 25 °C block temperature, 35 % relative air humidity, and 400 ppm CO_2 concentration (for more details see Junker et al., 2017). $IWUE_{GE}$ was calculated as the ratio of net CO_2 assimilation rate to stomatal conductance for water vapour. Light curves revealed that $IWUE_{GE}$ reached a plateau above a PPFD of 500 µmol photons m^{-2} s^{-1} in all provenances. Thus, $IWUE_{GE}$ was averaged for light intensities between 500 and 1500 µmol photons m^{-2} s^{-1}.

Isotopic measurements and calculations

All samples were ground and homogenized in liquid nitrogen. For isotope measurements ($\delta^{13}C$ and $\delta^{18}O$) in needle total organic matter (TOM), freshly frozen, ground aliquots of 0.15 g were dried and stored in desiccators. For isotope measurements ($\delta^{13}C$) in needle water-soluble organic matter (WSOM), freshly frozen, ground aliquots of 0.05 g were mixed with 1 ml of deionized water and agitated for 1 h at 4 °C (Gessler et al., 2009c; Ruehr et al., 2009). The extract was boiled at 100 °C for 3 min to precipitate proteins and centrifuged at 14 000 g for 5 min, decanted, and centrifuged again (2 min). The supernatant was considered to be the water-soluble, exportable fraction in the plant tissue consisting mainly of sugars but with some amino acids and organic acids, and was stored for further analyses. The isotopic composition of WSOM has proved to be an excellent proxy for sugars and thus recent assimilates (Gessler et al., 2009c) and is known to integrate IWUE over a period of hours to days (Keitel et al., 2003; Brandes et al., 2006).

For $\delta^{13}C$ and $\delta^{18}O$ analysis, the homogenised, oven-dried samples were combusted in a Flash HT elemental analyser (ThermoFinnigan, Bremen, Germany) coupled via a Conflo III interface to an isotope ratio mass spectrometer (Delta V Advantage, ThermoFisher Scientific GmbH, Bremen, Germany). Carbon and oxygen isotopic values were expressed in δ notation relative to the Vienna Pee Dee Belemnite (VPDB) and Vienna Standard Mean Ocean Water (VSMOW) standard, respectively.

The difference in altitude between the field sites Wiesloch and Schluchsee amounts to 945 m, thus we corrected the isotopic values for elevation effects: $\delta^{13}C$ values were corrected for the

effect of reduced O_2 partial pressure at higher elevation, which leads to an increase in $\delta^{13}C$ of on average 0.22 ‰ per 100 m (Körner et al., 1991; Keitel et al., 2006). $\delta^{18}O$ values were corrected for the temperature-controlled rain out effect that lowers $\delta^{18}O$ in precipitation with increasing altitude, which amounts to ~ -0.3 ‰ 100 m^{-1} (Sturm et al., 2005). For middle latitudes, there are no significant spatial variations in the elevation effect on $\delta^{18}O$ in precipitation (Poage & Chamberlain, 2001).

From the carbon isotope composition of the plant samples and the tropospheric CO_2 ($\delta^{13}C_{atm}$), we calculated the photosynthetic carbon stable isotope discrimination ($\Delta^{13}C$) according to Farquhar et al. (1982). $\delta^{13}C_{atm}$ follows a seasonal cycle with minimum values during the winter and a mean amplitude of 0.4 ‰ (Levin et al., 1995; Levin & Kromer, 1997a). For calculation of $\Delta^{13}C$, estimates of $\delta^{13}C_{atm}$ were based on averaged monthly data from long-term measurements at the station Schauinsland (48N, 8E , 1205m asl, Freiburg, Germany) between the years 1977-1997 (Levin & Kromer, 1997a,b), and corrected for a mean decrease in $\delta^{13}C_{atm}$ by 0.017 ‰ yr^{-1} and for an methodology based offset of 0.2 ‰ as reported by Levin and Kromer (1997a).

From $\Delta^{13}C$ determined for WSOM we calculated $IWUE_{13C}$ according to Farquhar et al. (1982) and Seibt et al. (2008). For this calculation we used monthly average tropospheric CO_2 concentrations that were obtained from long-term measurements (Umweltbundesamt, 2012) at the stations Schauinsland (48N, 8E , 1205 m asl) and Deuselbach (50N, 7E, 480 m asl).

Comparison between parameters derived from leaf gas exchange and isotopic composition

We compared $IWUE_{13C}$ in WSOM of previous-year needles with A_n, g_s, and $IWUE_{GE}$ measured on the same tissue and tree individuals (n=71) for the campaigns in May and July in the years 2010 and 2011. In July 2010, GE and $\delta^{13}C$ were measured at the site Wiesloch under similar environmental conditions on the Santiam River provenance, while this is not true for the other three provenances due to very variable weather conditions in this month. We therefore excluded GE data for the provenances Salmon Arm, Cameron Lake and Conrad Creek at Wiesloch in July 2010 (see Table S 3).

We derived time-integrated IWUE (over the two years 2010 and 2011) from: i) the average $\delta^{13}C_{TOM}$ of current-year needles sampled in September 2010 and 2011 ($IWUE_{13C\ TOM}$, Figure 5), and ii) the slope of the regression line of all measured A_n plotted against all measured g_s in which we consider the slope as an integrating IWUE over the whole measurement period ($IWUE_{GE\ slope}$). To evaluate how these time-integrated measures compare to short-term measures averaged over all time points in 2010 and 2011, we averaged iii) $\delta^{13}C_{WSOM}$ in previous-year needles ($IWUE_{13C\ WSOM\ AVG}$), and iv) the ratio of A_n and g_s assessed by GE measurements on previous-year needles ($IWUE_{GE\ AVG}$).

Leaf mineral and water content

To determine WC and MC, needle material was oven-dried (24 h, 105 °C). WC (%) is given as the difference between fresh and dry weight, relative to fresh weight. MC was determined after Kjeldahl digestion (Kjeldahl, 1883). For this purpose, 0.2 g needle powder was solubilized in 2.4 ml H_2O_2 and 2.4 ml selenium sulfuric acid at 380°C for 90 min, cooled down and filled up to 100 ml with H_2O. The solution was analysed for total N and P using the photometer AT200 (Beckman Coulter, Brea, CA, USA / Olympus, Tokyo, Japan) and for total K, Mg, Ca and Na using the spectrometer AAS-iCE3300 (ThermoFisher Scientific GmbH, Bremen, Germany). MC was calculated as the sum of N, P, K, Mg, Ca, Na contents relative to needle dry weight.

Statistical analyses

All measured parameters were characterized by descriptive statistics (means and standard deviations of the means). Statistical analyses were carried out with R 3.0.2 (R Core Team, 2013). Homogeneity of variances was tested with the Fligner-Killeen test on grouped data ($p>0.05$). Individually per field site, a 2-way analysis of variance was conducted to assess the effects of provenance, time point, and their interactions. ANOVA was performed on ranks if residuals showed non-normality. Normality of residuals was tested with the Shapiro-Wilk test ($p>0.1$). Tukey's post-hoc test following ANOVA was used for multiple comparisons among the groups. Correlations between parameters are based on Pearson's correlation coefficient and an F-test on the significance of the correlation.

2.4 Results

Comparison between parameters derived from leaf gas exchange and isotopic composition

$IWUE_{13C}$ revealed differences between field sites, time points and provenances. At Wiesloch, we found a significant effect of provenance on $IWUE_{13C}$ (Table S 1) but no significant differences in $IWUE_{13C}$ between specific time points. Salmon Arm and Cameron Lake showed significantly lower $IWUE_{13C}$ than Conrad Creek and Santiam River (Table S 2). At the cool humid site Schluchsee, differences in $IWUE_{13C}$ were stronger between time points than among provenances (Table S 1). The highest values in $IWUE_{13C}$ were generally observed in May and partly in July (Figure 4, Table S 2). The interior provenance Salmon Arm had significantly lower $IWUE_{13C}$ than the three coastal provenances (over all time points, Table S 2) while Cameron Lake showed the highest $IWUE_{13C}$.

Over all provenances, $IWUE_{13C}$ in May 2010 was significantly higher than in June, July and September 2010 at Schluchsee (Table S 2), a pattern not observed at Wiesloch. A strongly retarded bud development at Schluchsee (Junker et al., 2017) indicated an extreme delay in the growing season 2010 compared to 2011 and also to Wiesloch in 2010 . At a very early phase of the growing season not all leaf sugars originate directly from photosynthesis but are also mobilized from starch stored in the needles (c.f. Jäggi et al., 2002) most likely causing an uncoupling between the instantaneous IWUE measurements and the isotope based approach. Due to these particular conditions we see justification to exclude this time point at the Schluchsee site from further analyses.

In May 2011, we also observed significantly higher $IWUE_{13C}$ at Schluchsee (compared to September 2011, Table S 2). However, the vegetation period 2011 started without any delay but with exceptionally dry conditions as shown by Junker *et al.* (2017). We thus attribute the high IWUE values in May 2011 to the actual environmental conditions and include May 2011 in further analyses.

The offset between $IWUE_{GE}$ and $IWUE_{13C}$ amounted to 42.7±23.3 mmol CO_2 mol^{-1} H_2O over all trees at both sites, 52.1±20.1 mmol CO_2 mol^{-1} H_2O at Schluchsee and 32.5±22.4 mmol CO_2 mol^{-1} H_2O at Wiesloch (Table S 3).

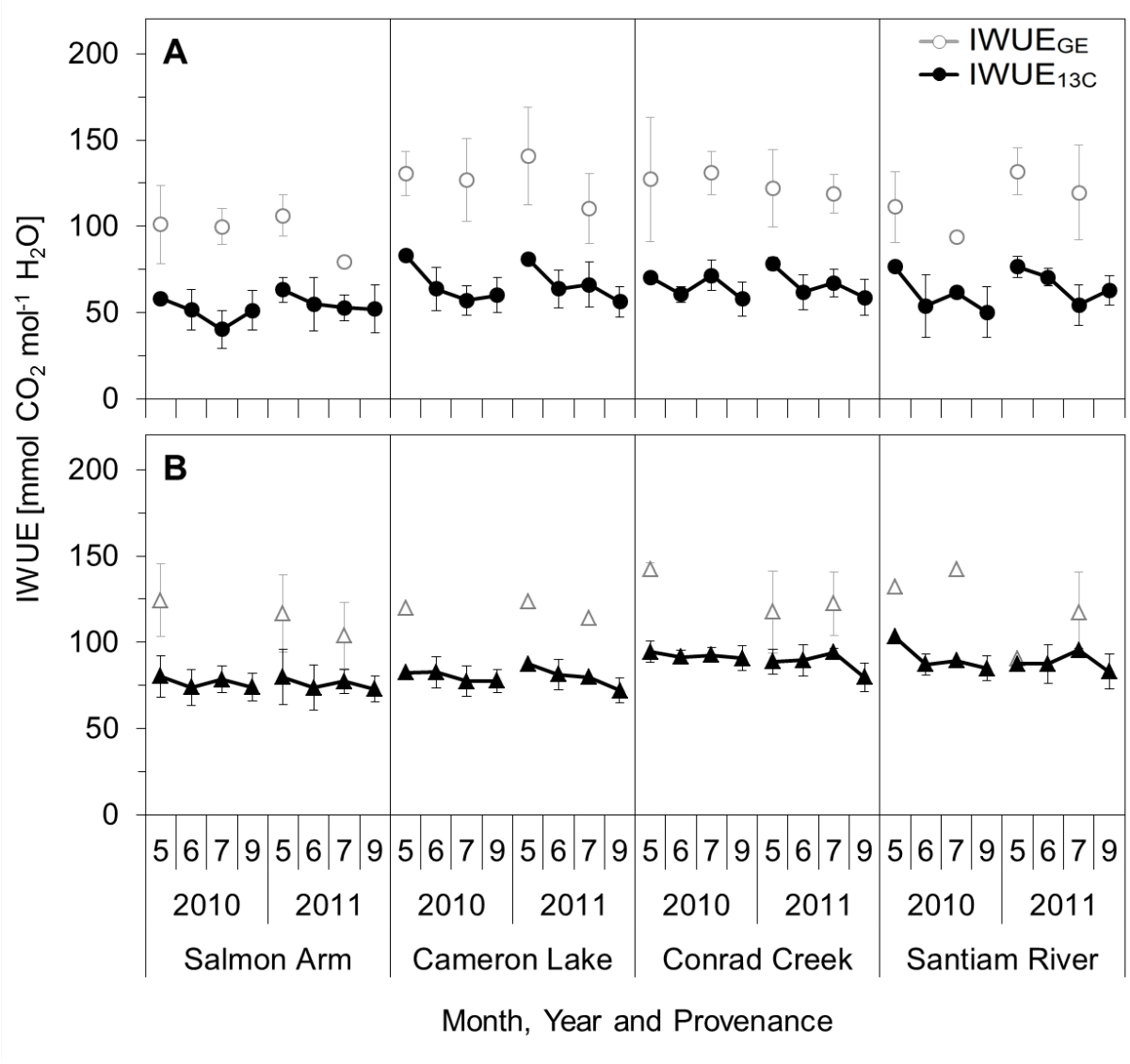

Figure 4 Intrinsic water-use efficiency derived from leaf gas exchange measurements ($IWUE_{GE}$, open symbols) and leaf carbon stable isotope composition (filled symbols, $IWUE_{13C}$) at the field sites Schluchsee (A, 1050 m asl) and Wiesloch (B, 105 m asl)
Previous-year needles were sampled in May (5), June (6), July (7) and Sep (9) of the years 2010 and 2011. Gas exchange data for three provenances in July 2010 at Wiesloch was excluded from all analyses (see method section). Water-soluble organic matter of the leaves was used for isotope measurements. Values shown are means ± SD (N=2-6)

When applying correlation analyses between $IWUE_{13C}$ and GE parameters (A_n, g_s, $IWUE_{GE}$, Table 7) the correlation was significant with each parameter at the Schluchsee but not significant at the Wiesloch site. The correlation at Schluchsee increased when the May 2010 values were excluded ($IWUE_{13C}$ vs. $IWUE_{GE}$, R^2=0.23, p=0.002 as displayed in Table 7), whereas the correlation was weakened when the May 2011 values were excluded instead (R^2=0.19, p=0.009, not shown).

Table 7 Correlation between isotope-derived intrinsic water-use efficiency ($IWUE_{13C}$) and parameters derived from gas exchange (A_n, g_s, WUE_{GE})
$IWUE_{13C}$ was derived from carbon stable isotope composition in water-soluble organic matter of the leaf. R (Pearsons correlation coefficient) and R^2 are bold in case of significance ($p < 0.05$)

			$IWUE_{GE}$	g_s	A_n
$IWUE_{13C}$	All sites	R	**0.32**	**-0.57**	**-0.52**
		R^2	**0.11**	**0.33**	**0.27**
		p	0.006	< 0.001	< 0.001
		N	71	71	71
$IWUE_{13C}$	Schluchsee	R	**0.48**	**-0.54**	**-0.45**
		R^2	**0.23**	**0.29**	**0.21**
		p	0.002	< 0.001	0.004
		N	38	38	38
$IWUE_{13C}$	Wiesloch	R	0.19	-0.11	-0.09
		R^2	0.04	0.01	0.01
		p	0.220	0.495	0.553
		N	43	43	43

We compared $IWUE_{13C\ TOM}$ and $IWUE_{GE\ slope}$, both integrating over the two years 2010 and 2011 (Figure 5), to short-term measures averaged over all time points in 2010 and 2011 ($IWUE_{13C\ WSOM\ AVG}$ and $IWUE_{GE\ AVG}$), $IWUE_{GE\ AVG}$ showed an average (positive) offset of 42.3 ± 11.6 mmol CO_2 mol^{-1} H_2O to $IWUE_{13C\ WSOM\ AVG}$, similar to the observed offset between $IWUE_{GE}$ and $IWUE_{13C}$ (42.7±23.3 mmol CO_2 mol^{-1} H_2O, see above).

Over both sites and all provenances, $IWUE_{13C\ WSOM\ AVG}$ and $IWUE_{13C\ TOM}$ were significantly positively correlated to each other (R^2=0.90, p=0.0003, n=8, not shown). These parameters showed a similar pattern amongst the provenances with Salmon Arm displaying the lowest IWUE at both sites (Figure 5). The correlation between the other parameters was not significant. At Wiesloch, correlations between gas exchange- and isotope-derived IWUE parameters were not significant whereas at Schluchsee, both $IWUE_{GE\ AVG}$ and $IWUE_{GE}$ slope were significantly correlated to $IWUE_{13C\ WSOM\ AVG}$ (R^2=0.91 and 0.92, p<0.05, n=4, not shown).

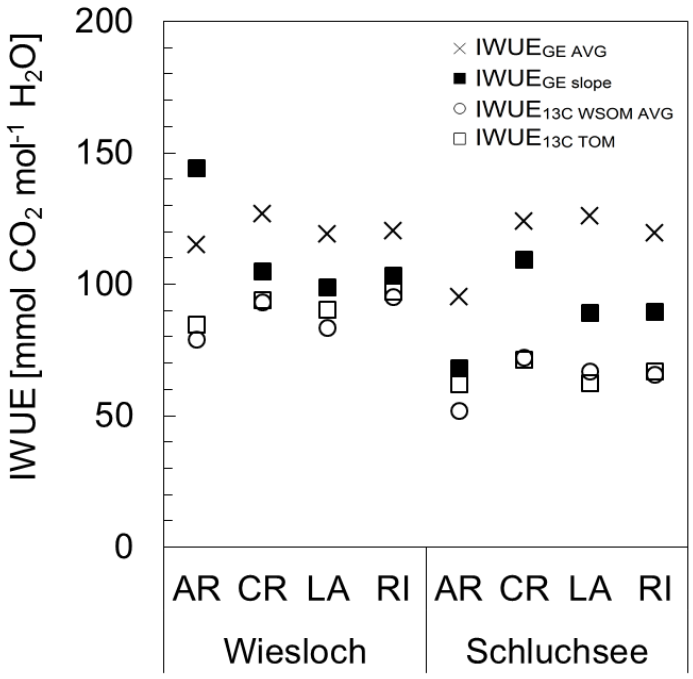

Figure 5 Intrinsic water-use efficiency (IWUE) derived from four different methods using leaf gas exchange (GE) measurements and leaf stable carbon isotope composition ($\delta^{13}C$)
The four provenances Salmon Arm (AR), Conrad Creek (CR), Cameron Lake (LA) and Santiam River (RI) were investigated at the field sites Wiesloch and Schluchsee. The data is based on 1) averages of single GE-based values of IWUE as the ratio between A_n and g_s ($IWUE_{GE\ AVG}$) determined in 2010 and 2011, 2) the slope of all measured A_n during 2010 and 2011 plotted against all measured g_s ($IWUE_{GE\ slope}$), 3) average $\delta^{13}C$ in water-soluble organic matter of needles ($IWUE_{13C\ WSOM\ AVG}$) determined over the years 2010 and 2011 and 4) $\delta^{13}C$ in needle total organic matter ($IWUE_{13C\ TOM}$) obtained in Sep 2010 and 2011. GE and $\delta^{13}C_{WSOM}$ were measured in May and Jul 2010 and 2011. Values from May 2010 at Schluchsee were excluded. Data shown are mean values (N=6-10) for $IWUE_{GE}$, $_{13CWSOM}$ and $_{13CTOM}$, and single values for $IWUE_{GE\ slope}$

The impact of environmental conditions on leaf stable isotope composition

To compare physiological responses between years, we applied the dual isotope approach according to Scheidegger et al. (2000) on TOM of current-year needles sampled in September 2010 and 2011: At Schluchsee, the provenance Cameron Lake displayed a decrease in $\delta^{13}C_{TOM}$ and $\delta^{18}O_{TOM}$ from 2010 to 2011 and the conceptual model from Scheidegger et al. (2000) thus points to an increase in g_s (Figure 6). The other three provenances showed a decrease in $\delta^{18}O_{TOM}$ but no clear change in $\delta^{13}C_{TOM}$. Thus, the Scheidegger model estimates a concomitant increase in A_{max} and g_s. At the valley site Wiesloch, Santiam River did not show differences in isotopic composition between the two years, while for Salmon Arm and Cameron Lake the dual isotope approach pointed to an increase in A_{max} from 2010 to 2011, and for Conrad Creek to concomitant increases in A_{max} and g_s.

We tested the correlation between $\delta^{13}C_{TOM}$ and weather conditions i) averaged over periods of 0-10 to 0-140 days before sampling in September (mid of September to the end of April, Figure 7A), as well as ii) averaged over 10-day periods between 0-10 and 130-140 days before sampling (Figure 7B).

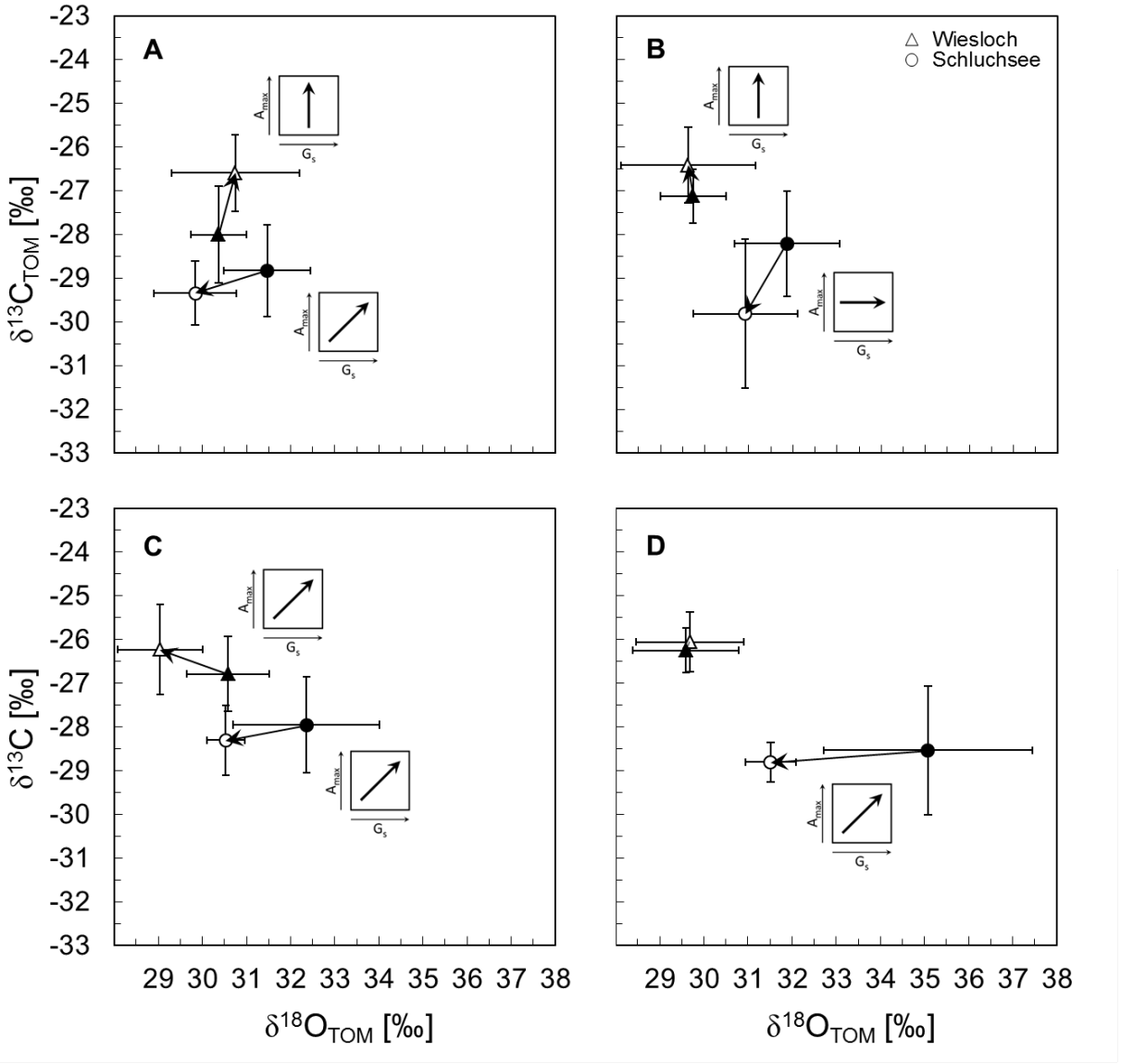

Figure 6 Physiological response to changing environmental conditions from 2010 (filled symbols) to 2011 (open symbols)
Stable isotope composition of carbon and oxygen in needle total organic matter ($\delta^{13}C_{TOM}$, $\delta^{18}O_{TOM}$) of the provenances Salmon Arm (A), Cameron Lake (B), Conrad Creek (C) and Santiam River (D) from the experimental sites Wiesloch (105 m asl; triangles) and Schluchsee (1050 m asl; circles). Current year's needles were sampled in September and the analysed TOM integrates over one growing season. The insets indicate how the Scheidegger et al. (2000) model translates changes in $\delta^{13}C_{TOM}$ and $\delta^{18}O_{TOM}$ to changes in stomatal conductance (g_s) or photosynthetic capacity (A_{max}). Data shown are means ± SD (N=5)

$\delta^{13}C_{TOM}$ was significantly positively correlated to daily maximum air temperature (T_{air}, Figure 7A). Daily minimum and average temperature showed similar results (data not shown). Air humidity (rH), precipitation sum (P), total available soil water (TAW) and sunshine hours (SH) were negatively correlated to $\delta^{13}C_{TOM}$. The correlation coefficient increased when weather conditions were averaged over longer periods (Figure 7A).

When averaging over 10-day periods, T_{air} showed strongest correlation with $\delta^{13}C_{TOM}$ from the end of May to the beginning of July, and TAW showed the strongest impact on $\delta^{13}C_{TOM}$ between the end of June and end of August (Figure 7B).

$\delta^{13}C_{WSOM}$ of needles sampled throughout the growing season (May (only Wiesloch), June, July and September 2010 and 2011) was correlated to environmental conditions averaged over one up to 20 days prior to sampling (Figure 8). At Schluchsee, $\delta^{13}C_{WSOM}$ was significantly negatively correlated to rH and P in most provenances (not significant in Salmon Arm, Figure 8A) and time spans prior to sampling, while at Wiesloch we found only significant correlation with SH for the provenance Cameron Lake (Figure 8B).

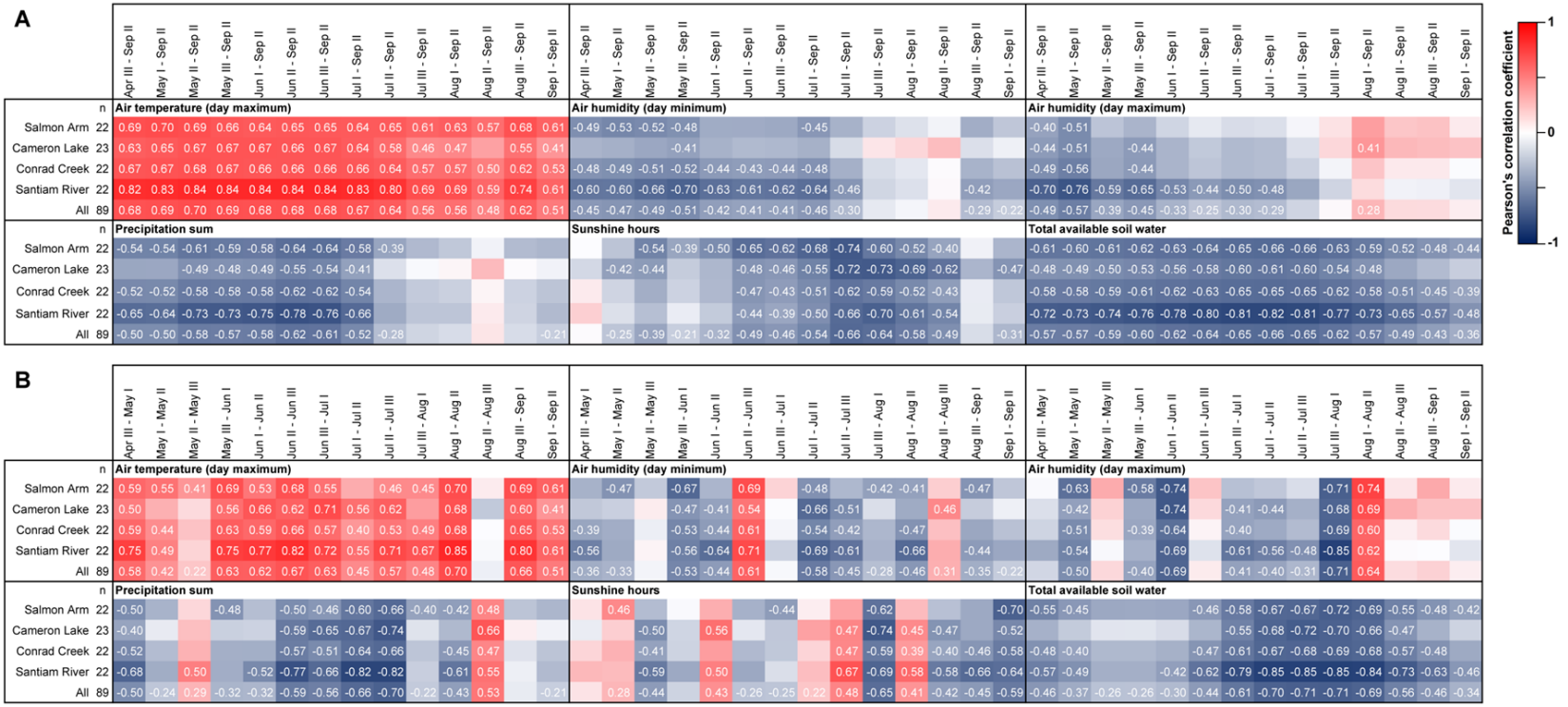

Figure 7 Physiological responses over the whole growing season: Correlation between leaf carbon stable isotope composition in total organic matter ($\delta^{13}C_{TOM}$) vs. weather conditions at the field sites Wiesloch and Schluchsee

Current-year needles were sampled in September 2010 and 2011 and are integrate over one growing season. Weather conditions were averaged over (A) 140-0 to 10-0 days before sampling and (B) averaged over 10-day periods from 131-140 to 0-10 days before sampling. The figure shows Pearson's Correlation Coefficient R (white numbers indicate a significance level of $p<0.05$)

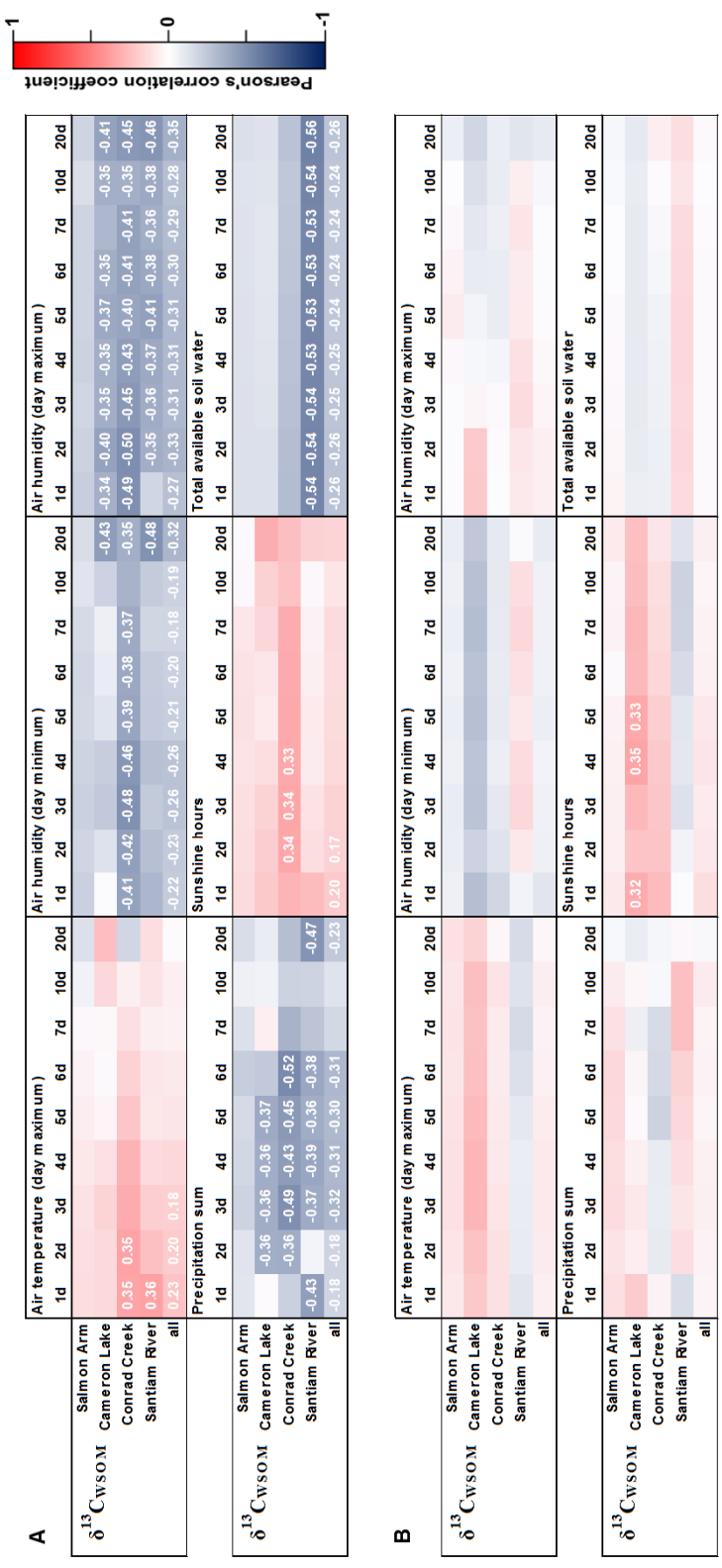

Figure 8 Correlation between carbon stable isotope composition in water-soluble organic matter (WSOM) of previous-year needles ($\delta^{13}C_{WSOM}$) vs. weather conditions averaged over 1 to 20 days before sampling at the field sites Schluchsee (A, 1050 m asl) and Wiesloch (B, 105 m asl)
Isotopic values from May 2010 at Schluchsee were excluded. The figure shows Pearson's Correlation Coefficient R (white letters for significance level $p<0.05$) correlated for each field site separately over 8 (Wiesloch) to 7 (Schluchsee) time points with 5 biological replicates each (May, June, July, Sep 2010 & 2011)

Relations between leaf carbon isotope composition, water content, and mineral content

In May and July in 2010 and 2011, respectively, we analysed leaf WC and MC, given as the sum of N, P, K, Mg, Ca, and Na content relative to dry weight. At Schluchsee, MC was significantly and positively correlated to WC over all provenances (R^2 = 0.14; p = 0.001, Figure S 1) but this was not the case at Wiesloch. As a consequence, the positive relationship of WC to rH was reflected by a comparable trend of MC at Schluchsee (Figure 9). $\delta^{13}C_{WSOM}$ was not significantly correlated to MC at both sites but at Schluchsee there was a negative correlation between $\delta^{13}C_{WSOM}$ and K which was also significant for two provenances (Figure S 2). In contrast to the results at Schluchsee, MC and WC were significantly negatively correlated to rH at Wiesloch (Figure 9, over all provenances). At this dry site, Santiam River was the only provenance showing a correlation between MC and TAW (R^2=0.87, p=0.066, data not shown), as well as a significant correlation between WC and MC (R^2=0.31, p=0.003, Figure S 1).

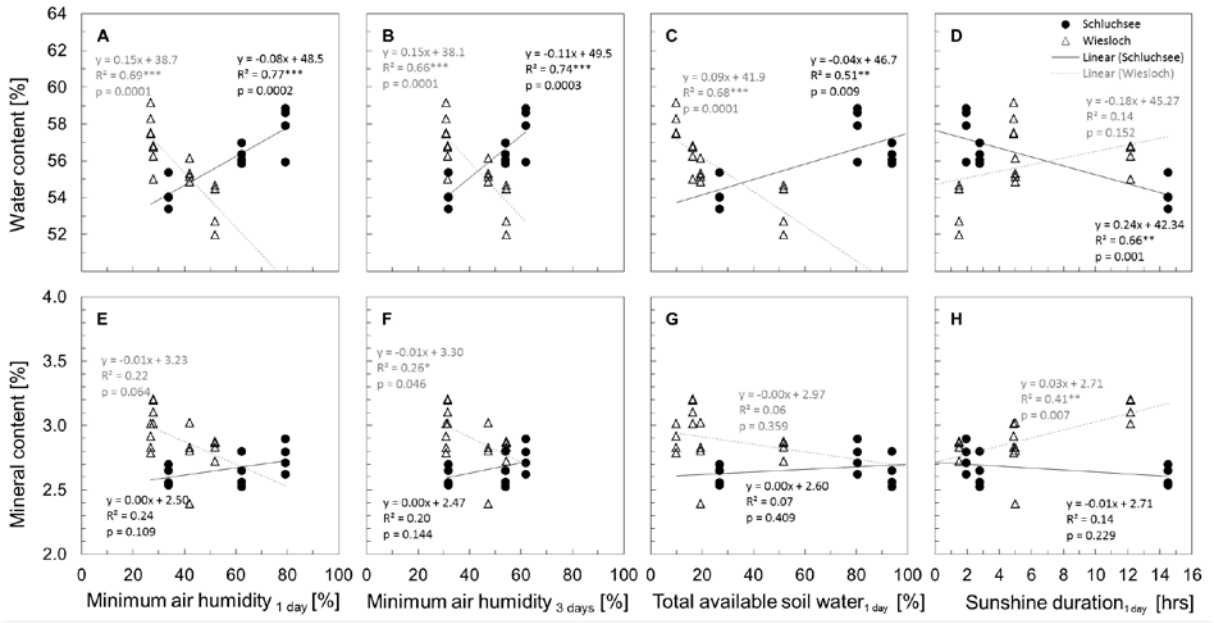

Figure 9 Correlation between weather conditions vs. leaf water content (A-D) and leaf mineral content (E-H) in previous-year needles

Weather conditions were averaged over 1 or 3 days before sampling. Data shown are mean values of 5 replicates per provenance, site and time point. Sampling took place in May and July 2010 and 2011 at Wiesloch and Schluchsee, under exclusion of May 2010 at Schluchsee. The figure shows Pearson's Correlation Coefficient R (asterisks indicate significance levels * p<0.05, ** p<0.01, *** p<0.001) for each field site separately (Wiesloch, triangles and dotted line, and Schluchsee, black circles and solid line)

2.5 Discussion

Parameters derived from leaf gas exchange and isotopic composition are related at the humid field site

$IWUE_{GE}$ is valid for the exact time point of leaf GE measurement only, whereas $IWUE_{13C}$, based on the isotopic signal in needle WSOM, integrates over a time period of up to 2 days (Keitel et al., 2003; Brandes et al., 2006). Hence, both techniques are highly complementary (as also observed at the humid site of this study) but might produce different results under particular conditions, for instance when GE measurement conditions and average ambient conditions differ, or when additional effects, such as changes in mesophyll conductance (g_m), play a role.

Indeed, we observed $IWUE_{13C}$ to be generally lower compared to $IWUE_{GE}$ (Figure 4) by on average 42.7 mmol CO_2 mol^{-1} H_2O (Table S 3). This offset might be due to i) post-photosynthetic carbon isotope fractionation that can lead to differences between $\delta^{13}C$ of primary assimilates and (non-reacting) leaf sugars (Hobbie & Werner, 2004) and consequently between $IWUE_{GE}$ and $IWUE_{13C}$. Leaf WSOM as a proxy for leaf sugars is, in most cases, enriched compared to primary assimilates (Gessler et al., 2008), and post-photosynthetic carbon isotope fractionation can cause an overestimation of IWUE by up to 20% in phloem sugars of Scots pine (Gessler et al., 2009a). Thus, we would expect $\delta^{13}C$-derived IWUE values to exceed the ones obtained from GE. We, however, observed the opposite pattern in this study and can thus rule out this mechanism. ii) GE measurements have been performed at defined light intensities > 500 µmol m^{-2} s^{-1}. Even though ambient light intensities were comparable for the needles analysed for isotopic composition, self-shading of the needles during parts of the day most likely reduced integrated light availability. Leverenz (1981) showed that A_n and g_s did not necessarily scale comparably with radiation in Douglas-fir and especially that g_s saturated at lower light intensities (200 µmol m^{-2} s^{-1}) than A. We might thus assume that especially under lower light conditions effective A_n/g_s differed from the $IWUE_{GE}$ values determined at PPFD > 500 µmol m^{-2} s^{-1}. In contrast, assimilates produced under low light conditions will be integrated into $IWUE_{13C}$. A significant contribution of assimilates produced during lower light intensities would thus explain the generally lower IWUE determined with isotope compared to the GE approaches. Furthermore, measurement temperature during GE was set to 25 °C

and rH fixed to 35 %, while ambient temperature was often lower and ambient rH was partly higher (both leading to lower effective IWUE). This was especially the case at Schluchsee, possibly explaining the larger offset between $IWUE_{GE}$ and $IWUE_{13C}$ compared to Wiesloch. iii) $\Delta^{13}C$ can be affected by changes in mesophyll conductance (g_m). $IWUE_{13C}$ is based on the estimation of $\Delta^{13}C$ by the simplified linear Farquhar model (Farquhar et al., 1982), which omits fractionation factors associated with g_m, assuming g_m more or less infinite. Leaves of evergreen trees have anatomical and biochemical traits associated with leaf longevity and drought resistance, which result in a high internal resistance to diffusion of CO_2 and consequently low g_m (Warren & Adams, 2004). Direct measurements of A_n, g_s and $IWUE_{GE}$ via gas exchange include potential variability in g_m but the linear Farquhar model might overestimate $\Delta^{13}C$, when g_m is finite and thus underestimate effective IWUE (Farquhar et al., 1989; Seibt et al., 2008). Bickford et al. (2010) showed such an effect also in the conifer Juniper (*Juniperus communis* L.). We thus conclude that the deviation of light intensity, temperature and rH between the cuvette measurements and average ambient conditions and the effects of g_m can explain the systematic difference between gas exchange- and isotope-based estimates of IWUE.

Despite this offset, we observed a close correlation between $IWUE_{13C}$ and GE parameters at Schluchsee (A_n, g_s, $IWUE_{GE}$, Table 7) , confirming our hypothesis (1). $IWUE_{13C}$ is negatively correlated to both, A_n and g_s, showing that the driving force for changes in c_i (and thus in $IWUE_{13C}$) is g_s and not A_n (in that case the correlation between $IWUE_{13C}$ and A_n should be positive).

In contrast to Schluchsee, we did not observe any correlation between $IWUE_{13C}$ and GE parameters (A_n, g_s, $IWUE_{GE}$, Table 7) at Wiesloch. For this field site, we cannot confirm that the information derived from the different methods is closely related to each other (hypothesis (1)) and must consider site-specific processes that might modify the relation between instantaneous and more integrative measures for IWUE: GE showed a small operational range of stomata with g_s and A_n at comparably low levels at Wiesloch (Junker et al., 2017), going in line with a low sensitivity of $\delta^{13}C_{WSOM}$ to environmental conditions (Figure 8). Therefore, a lack of correlation between $IWUE_{13C}$ and GE parameters at the Wiesloch site may be linked to the limited range of these parameters. We might also assume that a varying contribution of

low light assimilates (see above) to the sugar pools over the year caused an uncoupling between IWUE from the standardised GE measurements and the isotopic approaches.

The two isotope-derived, long term-integrating parameters $IWUE_{13C\ WSOM\ AVG}$ and $IWUE_{13C\ TOM}$ (Figure 5) were significantly positively correlated to each other over all field sites (data not shown) and thus reveal similar physiological information. At Schluchsee, the gas exchange-derived $IWUE_{GE\ AVG}$ and $IWUE_{GE\ slope}$ were significantly correlated to the isotope derived $IWUE_{13C\ WSOM\ AVG}$, reinforcing the observed correlation between instantaneous $IWUE_{GE}$ and $IWUE_{13C}$ (Table 7) and our hypothesis (1), and lead to the conclusion that isotope- and gas exchange-derived parameters for IWUE show consistent results at this humid field site. We cannot confirm hypothesis (1) for the dry site Wiesloch where neither the short nor the long term-integrating parameters derived from GE and needle isotopic composition were correlated to each other.

The impact of environmental conditions on leaf stable isotope composition differs between sites

$\delta^{13}C_{TOM}$ was derived from current-year needles that were sampled in September 2010 and 2011 and thus integrate over one growing season. rH, T_{air}, P, and TAW were negatively correlated to $\delta^{13}C_{TOM}$, when taking the two sites and the two years (2010 and 2011) together. The strongest correlations of environmental conditions with $\delta^{13}C_{TOM}$ were observed for all provenances between June and July (P), June and August (TAW), or May and August (T_{air}, Figure 7B) confirming hypothesis (2b). The assimilates produced during this period seem to contribute significantly to needle TOM as they convey the significant climatic imprint in the whole leaf isotopic signature. In current-year needles the structural carbon has been mainly laid down at the beginning of the growing season and is partially derived from storage pools. However, turn-over processes might lead to a continuous replacement by new assimilates (c.f. Gessler et al., 2007). Moreover, conifer needles are storage organs that provide carbohydrates for the early spring growth of the new needle flush (e.g. Fischer & Höll, 1991). Non-structural carbon compounds can contribute up to >20% of the needle/leaf biomass in trees (Hoch et al., 2003) and are thus most likely also involved in imprinting the isotopic signal on needle TOM during the current year. Our correlation analyses indicate that mainly assimilates produced in the summer months contribute to the total carbon isotopic signature of needles and thus the

needle storage pool rather than the structural carbon is responsible for this signal, which, consequently, reports mainly physiological responses of trees to environmental conditions during the summer months prior to sampling in September.

Leaf $\delta^{13}C_{WSOM}$ is a proxy for $\delta^{13}C$ in recent assimilates and provides short term integration of the physiological response of trees towards environmental conditions (Gessler et al., 2009c) and thus allows also for the assessment of seasonal variation of IWUE. At Schluchsee, $\delta^{13}C_{WSOM}$ was negatively correlated with rH, P and TAW, and positively correlated with SH and T_{air}. Most of these weather parameters showed that $\delta^{13}C_{WSOM}$ integrated over periods of days up to a couple of weeks (Figure 8) supporting hypothesis 2a. We furthermore observed differences in the strength of correlation between provenances. Salmon Arm, originating from a very dry region of origin, did not show any significant correlation between $\delta^{13}C_{WSOM}$ and environmental conditions at the field site Schluchsee. We thus confirm hypothesis (2c).

The fact that $\delta^{13}C$ reacts towards rH, P, and TAW at Schluchsee indicates that g_s (i.e. the stomatal response towards atmospheric and soil drought) is driving c_i over the short-term (as shown by the positive correlation between $IWUE_{13C}$ and g_s, Table 7). The dual isotope approach on leaf TOM corroborates these results as in all provenances at the site Schluchsee g_s is involved in affecting inter-annual differences in $\delta^{13}C$. Leaf GE, isotopic composition and the relation to weather parameters show concurrent results at the humid site.

At Wiesloch, however, parameters that are normally expected to affect g_s (P, rH, TAW) were not correlated with $\delta^{13}C_{WSOM}$ (Figure 8). This finding strongly disagrees with our hypothesis (2). At this site, $\delta^{13}C_{WSOM}$ was significantly correlated only with SH (that would affect rather A_n than g_s) in one provenance. When assessing the inter-annual differences with the dual isotope approach, changes in g_s were also not prominently determining the year-to-year variation in $\delta^{13}C$. GE measurements at Wiesloch (Junker et al., 2017) showed indeed a small operational range of stomata and we did not detect any correlation between $IWUE_{13C}$ and g_s (Table 7), showing that g_s had a rather weak effect on c_i. Similar to our findings, Srur et al. (2008) observed a stronger relationship between IWUE and soil water content at sites with generally good water availability compared to drier sites. Under the generally dry conditions as prevailing at the Wiesloch site, the relationship between weather cues and $\delta^{13}C$ might be modified by additional mechanisms.

Relations between leaf carbon isotope composition, water content, and mineral content

An increased leaf MC can lower the osmotic potential and thus cause increasing leaf WC, facilitating water uptake along the soil-plant gradient (Khan, 2000; Patakas et al., 2002; Silva et al., 2010). At Wiesloch, both, WC and MC increased significantly under drier conditions (Figure 9), indicating OA, thus confirming hypothesis (3). Moreover, OA in the needles of Douglas-fir growing at this dry field site is indicated by findings of Du et al. (prepared for publication) who reported more than three times higher contents of the organic osmolyte proline at Wiesloch compared to Schluchsee in needles sampled in July 2010. At Schluchsee, we did not find indicators for OA, and the observed negative correlation between $\delta^{13}C_{WSOM}$ and K (Figure S 2), and between $\delta^{13}C_{WSOM}$ and TAW (Figure 8) at this site might be associated to an increased uptake of minerals under high TAW.

$\delta^{13}C_{WSOM}$ was not significantly correlated to MC or K at the dry site Wiesloch (Figure S 2) matching the results of Masle et al. (1992) on plants exposed to drought, as well as our findings that $\delta^{13}C_{WSOM}$ was neither correlated to environmental conditions (Figure 8) nor under control of g_s (Table 8). At the same time, OA is indicated by our results at the dry site (Figure 9), potentially decoupling the link between $\delta^{13}C_{WSOM}$, g_s, and environmental conditions that we observed at the humid site. Martínez-Vilalta & Garcia-Forner (2016) demonstrated that across sites and species the control of leaf water potential is not necessarily linked to stomatal sensitivity, and OA has been shown to allow for maintaining photosynthesis under a gradual drying process (e.g. Shangguan et al., 1999).We conclude that OA might have affected leaf turgor and thus $\delta^{13}C$ of plant organic matter at the dry site Wiesloch. Consequently, we have to restrict hypothesis (2) which might not hold true under very dry conditions.

At Wiesloch, we also found some provenance-specific differences in OA that, in response to dry conditions, was most pronounced in Santiam River. Furthermore, the positive effect on leaf turgor (as indicated by the significant correlation between MC and WC, see Figure S 1) was clearly observed in Santiam River whereas in the other provenances this relation was not significant. We can confirm hypothesis (3a) insofar that OA was most pronounced in a provenance originating from a dry habitat of the coastal range. However, we cannot confirm pronounced OA in the interior provenance Salmon Arm from a very dry region of origin.

Our results on leaf $\delta^{13}C$, WC, and MC suggest that adult Douglas-fir growing at the low elevation site under warm and dry conditions maintain their (low) stomatal conductance and photosynthesis constant also under low soil water availability and/or low air humidity by strictly controlling the osmotic potential via the accumulation of minerals. We consider this the result of an acclimation process over the life span of the 50-year-old Douglas-fir trees. At the site, which is normally humid (Schluchsee), such a control was not evident, but the trees responded strongly to changing environmental conditions by altering stomatal conductance to avoid critical negative water potentials under soil or atmospheric drought.

2.6 Conclusions

We showed that mainly assimilates produced in the summer months (and thus the needle storage pool rather than the structural carbon) contribute to the total carbon isotopic signature of needles. Storage pools are assumed to be highly prioritised sinks (e.g. Dobbertin, 2005; Wiley & Helliker, 2012). Thus, current-year needle $\delta^{13}C$ could report physiological responses of trees to extreme events such as drought periods, when tree ring archives do not reliably provide information due to growth cessation (Pflug et al., 2015). Long-term stored needle material is available for many forest stands (e.g. Betson et al., 2007; Rautio et al., 2016) and might provide highly complementary information to tree ring isotope analyses.

Furthermore, we observed a strong coupling between water availability, leaf water content, stomatal conductance and carbon isotope discrimination in adult Douglas-fir trees growing at a humid site in Southwest Germany. This coupling is not clearly pronounced at a rather dry and warm site, where osmotic adjustment by accumulation of minerals during drought periods seems to be a major acclimation mechanism. At dry field sites, where stomatal conductance is low per se, the relationship between isotopic composition and weather parameters might not be interpreted straightforwardly and we need to take into account the effect of osmotic regulation on leaf physiology.

2.7 Acknowledgements

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2.8 Supplementary Information

Table S 1 Two-way ANOVA on stable carbon isotope composition in leaf water-soluble organic matter conducted per field site individually

Field site	Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Schluchsee	Provenance	3	23.65	7.88	8.64	0.00003	***
	Time point	7	65.13	9.31	10.20	0.00000	***
	Provenance X Time point	21	12.67	0.60	0.66	0.86300	
	Residuals	128	116.77	0.91			
Wiesloch	Provenance	3	49.20	16.40	27.17	0.00000	***
	Time point	7	7.44	1.06	1.76	0.10100	
	Provenance X Time point	21	6.91	0.33	0.55	0.94600	
	Residuals	128	77.26	0.60			

Table S 2 Post-hoc Tukey multiple comparisons of means on stable carbon isotope composition in leaf water-soluble organic matter

Field site	Comparisons	diff	lwr	upr	p adj	
Schluchsee	Cameron Lake - Salmon Arm	0.88	0.37	1.38	0.00007	***
	Conrad Creek - Salmon Arm	0.73	0.23	1.24	0.00120	**
	Santiam River - Salmon Arm	0.63	0.13	1.13	0.00726	**
Schluchsee	June 2010 - May 2010	-1.14	-1.98	-0.30	0.00137	**
	July 2010 - May 2010	-1.16	-1.99	-0.32	0.00107	**
	July 2011 - May 2010	-0.88	-1.72	-0.04	0.03152	*
	Sep 2010 - May 2010	-1.33	-2.17	-0.49	0.00008	***
	Sep 2011 - May 2010	-1.14	-1.98	-0.30	0.00139	**
	June 2010 - May 2011	-1.00	-1.83	-0.16	0.00865	**
	July 2010 - May 2011	-1.01	-1.85	-0.17	0.00691	**
	Sep 2010 - May 2011	-1.19	-2.03	-0.35	0.00063	***
	Sep 2011 - May 2011	-1.00	-1.83	-0.16	0.00872	**
Wiesloch	Conrad Creek - Salmon Arm	1.31	0.86	1.76	0.00000	***
	Santiam River - Salmon Arm	1.12	0.67	1.57	0.00000	***
	Conrad Creek - Cameron Lake	1.05	0.60	1.50	0.00000	***
	Santiam River - Cameron Lake	0.87	0.41	1.32	0.00001	***
Wiesloch	no significant differences					

Table S 3 Intrinsic water-use efficiency derived from stable carbon isotope composition in leaf water-soluble organic matter ($IWUE_{13C}$) and from gas exchange measurements ($IWUE_{GE}$)

Field site Provenance	Month	Year	N	$IWUE_{GE}$ (mmol CO_2 mol-1 H_2O ± SD)	$IWUE_{13C}$ (mmol CO_2 mol-1 H_2O ± SD)	Offset (mmol CO_2 mol-1 H_2O ± SD)
Wiesloch						
All provenances	All months	All years	34	120.58 ± 21.47	88.11 ± 11.60	32.47 ± 22.37
Salmon Arm	May	2010	3	124.32 ± 21.09	80.17 ± 14.61	44.15 ± 24.33
	May	2011	3	116.61 ± 22.37	79.36 ± 19.57	37.25 ± 19.94
	July	2011	3	103.84 ± 19.12	77.22 ± 6.85	26.62 ± 14.25
Cameron Lake	May	2010	2	119.80 ± 27.40	82.38 ± 9.17	37.41 ± 36.57
	May	2011	2	123.59 ± 49.22	87.63 ± 9.20	35.96 ± 58.42
	July	2011	2	113.80 ± 10.83	79.82 ± 11.16	33.98 ± 0.33
Conrad Creek	May	2010	3	142.34 ± 3.88	94.45 ± 6.01	47.89 ± 9.84
	May	2011	3	117.59 ± 23.84	89.89 ± 8.31	27.70 ± 23.79
	July	2011	4	122.38 ± 18.25	94.23 ± 2.32	28.16 ± 19.59
Santiam River	May	2010	2	131.97 ± 19.08	107.62 ± 4.11	24.35 ± 14.97
	July	2010	2	142.26 ± 10.01	89.37 ± 10.76	52.89 ± 20.77
	May	2011	2	90.60 ± 7.91	87.55 ± 17.56	3.05 ± 25.47
	July	2011	3	117.33 ± 23.23	95.58 ± 0.61	21.74 ± 22.81
Schluchsee						
All provenances	All months	All years	37	116.38 ± 22.81	64.28 ± 13.51	52.10 ± 20.10
Salmon Arm	May	2010	3	101.09 ± 22.77	58.20 ± 18.83	54.38 ± 7.62
	July	2010	3	99.85 ± 10.27	40.26 ± 11.02	59.59 ± 8.86
	May	2011	3	106.30 ± 11.77	63.11 ± 7.13	43.19 ± 18.88
	July	2011	3	79.61 ± 2.78	52.68 ± 7.29	26.93 ± 6.56
Cameron Lake	May	2010	3	130.57 ± 12.63	83.21 ± 18.43	47.36 ± 25.91
	July	2010	4	126.60 ± 24.03	56.99 ± 8.35	69.61 ± 24.20
	May	2011	3	140.72 ± 28.09	81.12 ± 2.53	59.60 ± 25.76
	July	2011	3	110.45 ± 20.24	66.09 ± 13.02	44.36 ± 15.58
Conrad Creek	May	2010	3	127.23 ± 36.12	70.46 ± 9.55	56.76 ± 35.29
	July	2010	3	130.91 ± 12.36	71.61 ± 8.75	59.29 ± 21.08
	May	2011	3	121.93 ± 22.28	77.43 ± 3.98	44.50 ± 22.97
	July	2011	3	118.84 ± 11.07	67.13 ± 7.90	51.71 ± 16.17
Santiam River	May	2010	3	111.29 ± 20.49	76.56 ± 11.32	34.73 ± 10.63
	July	2010	2	93.96 ± 17.13	61.72 ± 6.94	32.23 ± 10.18
	May	2011	4	131.82 ± 13.50	76.46 ± 6.12	55.36 ± 17.25
	July	2011	3	119.55 ± 27.32	54.32 ± 11.57	65.22 ± 22.29

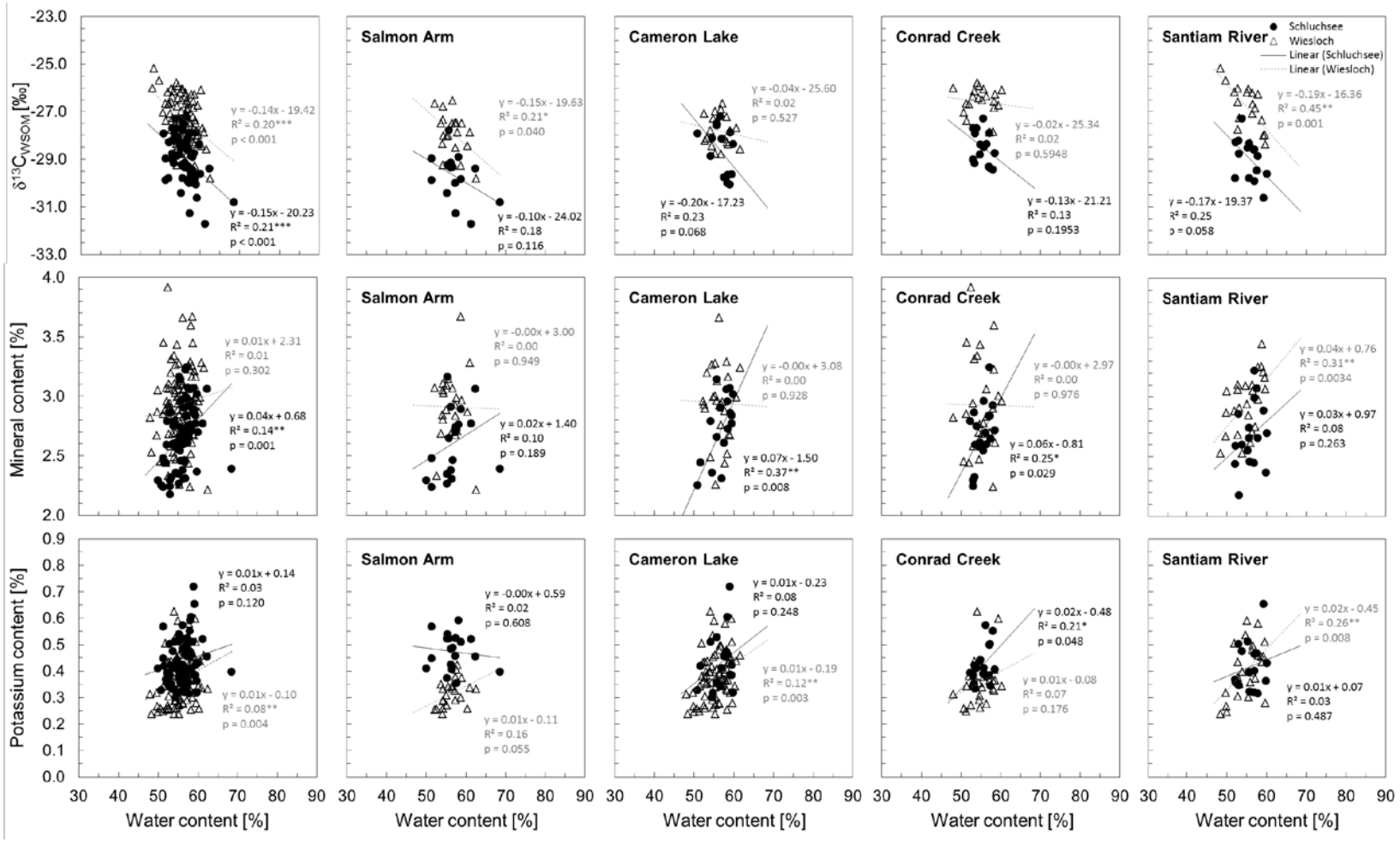

Figure S 1 Relation between carbon isotope composition in water-soluble organic matter of the leaf ($\delta^{13}C_{WSOM}$), mineral, potassium and water content

Previous-year needles of four provenances were sampled at Wiesloch and Schluchsee in May and July 2010 and 2011, under exclusion of May 2010 at Schluchsee. $\delta^{13}C$ was analysed in the water-soluble organic matter (WSOM) of the leaves

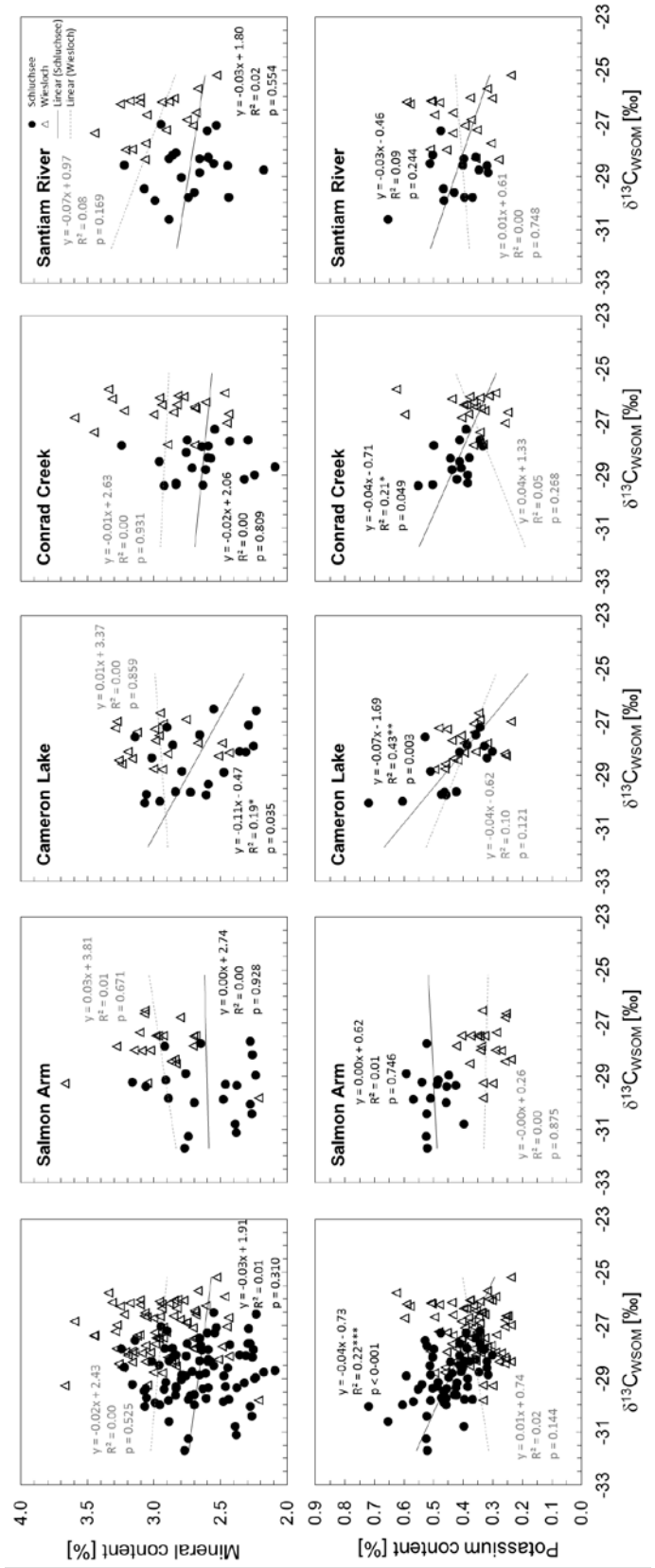

Figure S 2 Leaf mineral and potassium content versus carbon isotope composition in leaf water-soluble organic matter ($\delta^{13}C_{WSOM}$) in four provenances
Previous-year needles were sampled at Wiesloch and Schluchsee in May and July 2010 and 2011, under exclusion of May 2010 at Schluchsee

3 Tree Ring Isotopic Composition and Growth in Douglas–fir

Title of the publication: Tree ring isotopic composition, radial increment and height growth reveal provenance-specific reactions of Douglas-fir towards environmental parameters

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3.1 Abstract

In the search of timber species being tolerant towards summer droughts, which are expected to be more frequent in future, Douglas-fir is often discussed as a potential alternative for spruce in Central Europe. To assess physiological and growth reactions of Douglas-fir provenances towards climate- and weather-related environmental conditions we took advantage of a provenance trial with three sites in south-western Germany located along an elevation gradient. We examined six different provenances of Douglas-fir from North America for oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) stable isotope composition in tree rings as well as for radial increment for a 7 year period and long-term height growth. Our results show that different Douglas-fir provenances clearly vary in their drought sensitivity at the driest and warmest site in the valley as shown by the radial growth decline in the extreme dry and hot year 2003. The growth decline in the provenances Pamelia Creek, Cameron Lake, Duncan Paldi and Conrad Creek could be clearly attributed to a reduction in stomatal conductance as assessed by the relations between $\delta^{18}O$ and $\delta^{13}C$ in the tree rings. These responses were not related to the long-term average climate at the places of origin of the provenances and the provenances with the lowest long-term (height) growth potential were the ones least affected in radial increment by the extreme drought of 2003. When selecting suitable Douglas-fir provenances, which are adapted to the climatic conditions projected for the future we thus might need to take into

account the trade-off between the adaptation to extreme drought periods and the long-term growth performance. Site-specific evaluations of the probability of extreme drought events are thus needed to select the appropriate provenances.

3.2 Introduction

Global climate change is expected to affect tree growth, physiological performance and the distribution of species in temperate forest ecosystems in future (IPCC, 2007). One key for understanding the effect of climatic variables on plant performance over the longer term and retrospectively is to characterise and interpret the physiological information laid down in natural archives. For woody plants with secondary growth the tree rings provide an easily datable archive that can be explored for physiologically relevant signals (Leavitt, 1993; Hattenschwiler *et al.*, 1996; Livingston & Spittlehouse, 1996; Saurer, 2003; McCarroll & Loader, 2004). In addition to the assessment of 'traditional' growth traits such as radial or basal area increment, stable isotope ratios in tree ring cellulose or whole wood are increasingly characterised to obtain retrospective information to understand ecophysiological processes and their response to changing environmental conditions (Saurer *et al.*, 1997; Schleser *et al.*, 1999; Treydte *et al.*, 2001, 2006; Leavitt, 2002; Poussart *et al.*, 2004; McCarroll & Loader, 2004).

The carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope composition of organic matter in plant tissues depends on the one hand on the isotopic composition of the inorganic source. The source is the atmospheric CO_2 taken up by and assimilated in the leaf for $\delta^{13}C$ and the water taken up from the soil by the roots and transported to the leaf via the xylem for $\delta^{18}O$. On the other hand, isotope fractionation processes associated with diffusion, dissolution and phase transition as well as with enzyme reactions in the plant alter the original isotope signal of the source and thus imprint physiological information on the isotope composition of newly assimilated organic matter (Farquhar *et al.*, 1982; Brugnoli *et al.*, 1988; Cernusak *et al.*, 2005). Incorporation of recent assimilates into the tree-ring structural organic matter transfers significant portions of the original isotope signal to the tree-ring archive (Gessler *et al.*, 2009a).

The photosynthetic isotope fractionation ($\Delta^{13}C$) and thus organic matter $\delta^{13}C$ is an indicator for the ratio of ambient (c_a) to leaf intercellular (c_i), and more precise, to chloroplastic (c_c) CO_2 concentration (Farquhar *et al.*, 1982). Since c_i/c_a and c_i/c_c are strongly dependent on both, stomatal conductance (g_s) and photosynthetic assimilation capacity (A_n), $\delta^{13}C$ has been used as an indicator of intrinsic water-use efficiency (IWUE = A_n/g_s) (Farquhar *et al.*, 1989).

The oxygen stable isotope composition ($\delta^{18}O$) of plant organic matter provides additional physiological information to distinguish effects of stomatal conductance (as affected by water availability/air humidity) from effects of changes in photosynthetic capacity (as influenced by irradiance, temperature and nutrient availability) on the $\delta^{13}C$ (Farquhar et al., 1998; Scheidegger et al., 2000; Gessler et al., 2009a). This is due to the fact that the evaporative enrichment ($\Delta^{18}O$) of leaf water is mainly determined by the ratio (e_a/e_i) of the water vapour pressure in the atmosphere (e_a) and the leaf intercellular air space (e_i). The isotopic signal of evaporative enrichment of leaf water is imprinted with an equilibrium fractionation factor of 27 ‰ (Sternberg & DeNiro, 1983; Cernusak *et al.,* 2005) on the carbonyl group of organic matter. Since the water pressure ratio is also a major driver of stomatal conductance, $\delta^{18}O$ is related to stomatal conductance like $\delta^{13}C$ but, in contrast, is not influenced by variations in A_n (Barbour *et al.*, 2000a; Scheidegger *et al.*, 2000). Based on these relations Scheidegger *et al.* (2000) developed a conceptual dual isotope model, which allows evaluating if changes in A_n or in g_s (or in both) are responsible for changes in $\delta^{13}C$ and thus assessing the physiological performance of trees under drought from tree rings retrospectively.

Throughout Europe, the important timber species are usually managed in rather long rotation periods. Although extreme weather events are rare, they may affect growth and timber production considerably. As extreme events such as severe drought are predicted to occur more frequently in future in Central Europe (IPCC, 2007), timber production might be at risk and economical losses may increase. Norway spruce, currently the economic staple tree species in Central Europe is rather drought sensitive (Mäkinen *et al.*, 2001; Gaul *et al.*, 2008; Ge *et al.*, 2010; Lebourgeois *et al.*, 2010) and its productivity is likely to be compromised by climate change (Hanewinkel *et al.*, 2010; Teuffel, 2010). Therefore, more tolerant replacement species are sought for particular areas where high risks for summer droughts are to be expected in future.

In this context, Douglas-fir (*Pseudotsuga menziesii*), is often discussed as a potential commercial alternative to substitute anticipated decreased timber production by spruce. The species is native in America's Pacific Northwest (USA, Canada) and has been introduced in Central Europe at the end of the 19th century. Since then, Douglas-fir has become a highly valued commercial tree species in Europe. Major reasons are the species' outstanding growth and economic potential (Brandl, 1989; Heidingsfelder & Knoke, 2004). The natural range of the coastal variety of Douglas-fir (var. *menziesii*) extends in North America from north to south well over 2,000 km from the rather wet coastal climates in the Pacific Northwest's fog-belt and the adjacent summer-dry Coast Range and Cascade Mountains (BC, WA, OR) to the even drier coastlands of southern California. The interior variety (var. *glauca*) extends over 4,500 km along the dry continental climates of the montane to the subalpine Rocky Mountains from Alberta to Colorado. The species grows from sea level up to elevations of ca. 3,000 m asl (Hermann & Lavender, 1999).

Within its natural range, Douglas-fir has evolved a large variety of genetically diverse populations adapted to rather contrasting ecozones (e.g. Campbell, 1991; Dean, 2007; Gugger *et al.*, 2010). The large genetic pool of the locally well-adapted Douglas-fir provenances in North America has always attracted researchers to select suitable provenances to grow in diverse European climates (Kleinschmit & Bastien, 1992). However, none of these earlier projects ever aimed to select provenances suitable to grow and persist under the anticipated rapidly changing future climatic conditions.

The present study was focussed on assessing growth and physiological reactions (indicated by stable isotopes in tree rings) of Douglas-fir taking advantage of a provenance trial that was started in the early 1960s in south-western Germany (Kenk & Thren, 1984). At three well-documented experimental field sites of this trial series, ca. 50 years old (adult) Douglas-fir of six provenances (growing at each site) were examined. The aim of the study was to (1) identify provenance-specific responses of height and radial growth along an altitudinal and thus a temperature and precipitation gradient and (2) assess canopy physiology retrospectively by characterising the $\delta^{13}C$ and $\delta^{18}O$ isotope composition in the latewood of the tree rings between 2002 and 2007. In the retrospective part of the study, we focussed on the hot and dry year 2003 (Bréda *et al.*, 2006) in order to characterise physiological and radial growth responses towards extreme summer drought.

We hypothesised that local adaptation in the original habitat results in provenance-specific differences of drought sensitivity and can be detected by changes in IWUE and stomatal conductance combined with differences in growth. We also hypothesised that provenances originating from regions with lower precipitation are less affected by extreme drought periods than provenances from regions with higher precipitation.

3.3 Materials and methods

Experimental sites and provenance trial

The trees analysed in this study were collected from three different experimental field sites in the vicinity of Freiburg, Germany. The field sites are situated at elevations of 230 m asl (Dgl115 Mooswald), 500 m asl (Dgl114 Illenberg), and 940 m asl (Dgl116 Schauinsland) and cover a considerable gradient of precipitation and temperature characteristics (Table 8). At all three sites, nine coastal Douglas-fir provenances from different geographical seed source origins were grown. In the present study, we examined six of these provenances, originating from climatically contrasting environments covering a growing season rainfall gradient from 220 mm (Duncan Paldi) to 518 mm (Conrad Creek) (Table 9). The six selected provenances cover the full extent of the North American north–south as well as the west–east range of the seed source origins of all nine provenances present at the three experiment locations.

Table 8 Characteristics of the three experimental sites near Freiburg (Germany). Average sums of precipitation and mean temperature calculated for the period 1971 – 2007

Site	Elevation (m asl)	Precipitation (mm)		Mean temperature (°C)	
		Years	May-October	Years	May-October
Mooswald (Dgl 115)	230	823	495	10.7	16.3
Illenberg (Dgl 114)	500	1170	652	9.1	14.7
Schauinsland (Dgl 116)	940	1639	858	6.6	11.9

For the three different sites, several variables describing weather characteristics of the years 2002–2007 were calculated from monthly resolved data on temperature, precipitation and sunshine hours. The monthly data were based on weather records obtained from measurement stations of Deutscher Wetterdienst (DWD), which had been modelled for continuous spatial

resolution by Max-Planck-Institute for Meteorology (Hamburg) based on the principles of the REMO regional climate model (e.g. Jacob *et al.*, 2007; Jacob & Lorenz, 2009). In our analyses, we included the following variables describing weather characteristics: sum of precipitation (P_{sum}), maximum temperature (T_{max}), minimum temperature (T_{min}), average temperature (T_{mean}), and sum of sunshine hours (Sun_{sum}). For the different years of the investigation, the monthly values of these variables were either used as distinct characteristics for single months (June, July, or August, respectively) or aggregated for the following periods: January–December (year), growing season (April–September), or summer (June–July), respectively.

Table 9 Geographical seed source origin of the North American Douglas-fir provenances growing at the three experimental field sites near Freiburg (Germany) according to Kenk and Thren (1984). Temperature and precipitation are long-term annual or growing season means and sums, respectively

Province / State	Region[1]	Provenance	Elevation (m asl)	West. longitude	North. latitude	Temperature (°C)		Precipitation (mm)	
						year	growing season	year	growing season
BC	Vancouver Island	Cameron Lake	210	124°40'	49°15'	10.0	15.0	1475	320
		Duncan Paldi[2]	260	123°50'	48°45'	11.0	15.0	990	220
WA	North Cascades	Conrad Creek	280	121°30'	48°15'	9.5	14.0	2300	518
OR	West Cascades	Pamelia Creek	750	121°50'	44°40'	9.5	14.5	1780	410
		Santiam River	800	121°58'	44°40'	9.5	14.5	1780	410
	Coast Range	Timber	270	123°23'	45°48'	10.0	14.0	2390	358

1 USA: physiographic regions according to Franklin & Dyrness (1973); *2 synonym*: Darrington3

The three experimental field sites included in our study were part of a series of provenance experiments (Kenk & Thren, 1984). These experiments had been established in the early 1960s (Strehlke, 1959). At all three sites, the plots had been planted at a density of ca. 3,300 trees ha^{-1}: each provenance was planted in two replicate plots à 0.1 ha at Dgl115 and Dgl114, and in one plot à 0.04 ha at Dgl116. The first thinning was performed at a stand height of 10–12 m and uniformly reduced the stand density to 1,400 trees ha^{-1} by removing mostly small diameter trees. Simultaneously, 150 crop trees ha^{-1} were permanently selected, trying to accommodate adequately the aspects vigour, quality, and spacing. The second thinning was conducted at a stand height of 18 m and was designed as selective high thinning in favour of the selected crop trees. Since then, thinnings have been repeated approximately every 5 years. At each entry, stand density was reduced by selective high thinning to the height-specific tar-

get densities specified by the stand density curve "starke Durchforstung" (i.e. intensive thinning) developed for Douglas-fir in Southwest Germany by Kenk and Hradetzky (1984).

Field campaign and sampling

A regular measurement and treatment campaign was carried out during the winter 2007/2008 when stand ages were 51 years. For the 100 thickest trees ha^{-1} the average height (h_{100}) ranged among the provenance plots from 31.7 to 34.6 m (Dgl115), 29.1–33.3 m (Dgl114), and 29.3–31.3 m (Dgl116); the average diameter (d_{100}) at breast height (1.3 m) ranged from 42.2 to 51.1 cm (Dgl115), 44.4–51.6 cm (Dgl114), and 43.5–48.8 cm (Dgl116). From the trees removed during the scheduled thinnings, crosssectional discs were cut from the stems at breast height. After drying, the width of the annual rings from 2002 to 2007 was measured on 8 radii on each disk from four trees per provenance. Average width of the tree rings was calculated and used as indicator for a tree's annual radial increment in the respective year.

Thereafter the latewood of 3 radii per tree was sampled with chisels and scalpels. The material was homogenised with a ball mill, dried at 65 °C in an oven and subjected to isotope measurements.

For 12 latewood samples (6 provenances (Dgl 114; Illenberg site) × 2 years [2003 and 2002]) we divided the material in two subsamples. One of these subsamples was subjected to cellulose extraction according to Brenninkmeijer (1983) before isotope measurement. Cellulose was on average (for all samples) enriched in ^{13}C by 2.9 ± 0.3 ‰ and in ^{18}O by 4.3 ± 0.4 ‰ compared to the whole wood. The isotopic offset between whole wood and cellulose for both, $\delta^{18}O$ and $\delta^{13}C$, was not significantly different between the 2 years as determined with a paired samples t test. As a consequence we conclude that the whole wood isotopic signature is well suited to compare intra-annual differences in canopy physiology as affected by environmental conditions.

Isotope measurements and calculations

For $\delta^{13}C$ analysis, the homogenised samples were combusted in an elemental analyser (NA 2500; CE Instruments, Milan, Italy) and for $\delta^{18}O$ analysis in a high temperature conversion/elemental analyser (TC/EA; Finnigan MAT GmbH, Bremen, Germany), both coupled to

isotope ratio mass spectrometers (Delta Plus or Delta Plus XP, Finnigan MAT GmbH, Bremen, Germany) by a Conflo II/III interface (Finningan MAT GmbH, Bremen, Germany). Carbon isotopic values were expressed in δ notation relative to the Vienna Pee Dee Belemnite (VPDB) standard. For oxygen, δ notation, relative to the Vienna Standard Mean Ocean Water (VSMOW) was used. In order to avoid absorption of water vapour to the dried plant sample material and thus an artificial alteration of the $\delta^{18}O$ value (c.f. Cernusak *et al.*, 2003; Brandes *et al.*, 2007), we shielded all samples under an argon atmosphere and kept the autosampler of the TC/EA flushed with dry argon. The precision for measurements of both isotopes as determined by repeated measurements of standards (n = 10) was better than 0.1 ‰.

From $\delta^{13}C$ of the tree ring samples ($\delta^{13}C_{plant}$) we calculated the photosynthetic carbon isotope fractionation ($\Delta^{13}C$) according to the following equation:

$$\Delta^{13}C = \frac{\delta^{13}C_{atm} - \delta^{13}C_{plant}}{1 + \frac{\delta^{13}C_{plant}}{1000}} \qquad \text{Equation 1}$$

$\delta^{13}C$ of tropospheric CO_2 ($\delta^{13}C_{atm}$) was assumed to amount to 8 ‰ as growing season average (Farquhar *et al.*, 1989; Levin *et al.*, 1995). Intrinsic water-use efficiency (IWUE) as the ratio between assimilation rate (A_n) and stomatal conductance (g_s) was calculated from $\Delta^{13}C$ according to Equation 2 (c.f. Farquhar *et al.*, 1982; Seibt *et al.*, 2008):

$$IWUE_{13C} = \frac{c_a}{1.6}\left(\frac{b - \Delta^{13}C}{b - a}\right) \qquad \text{Equation 2}$$

where a is the fractionation during diffusion through stomata and leaf intercellular space and b is the carbon isotope discrimination during carboxylation by ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) (Farquhar *et al.*, 1982). The ambient CO_2 concentration (c_a) was calculated as a growing season average value from data collected between 1977 and 1992 in close vicinity to our field sites (Levin *et al.*, 1995). Only recently we have shown that post-photosynthetic carbon isotope discrimination may significantly alter the carbon isotopic composition of organic matter after assimilation and associated with transport of sugars in the phloem down the trunk (Gessler *et al.*, 2009a). As a consequence, the absolute 'real' values of IWUE might be overestimated by up to 20 % by tree ring carbon isotope approaches as applied here (see discussion in Gessler *et al.*, 2009a). We thus restricted our analysis of IWUE

to assessment of changes of IWUE between years, which should be not affected by the abovementioned processes.

Analysis of long-term height growth

To investigate possible differences in long-term growth between the provenances, we chose height as indicator for the trees' growth potential. In contrast to diameter growth, which is significantly affected by intra-stand competition, height growth is only marginally affected by competition and is used for growth analysis without the application of complex analytical approaches necessary in diameter-based growth analysis (Yue *et al.*, 2011).

We used the data on tree height recorded during the last periodic measurement of the stands after the termination of the growing season 2007 (age 51 years). In order to provide the basis for constructing stand height curves, an average of 23 trees per plot (min–max 9–40) was measured on each of the 30 plots (Mooswald: 12 plots, Illenberg: 12 plots, Schauinsland 6 plots). The trees selected for height measurement were chosen across the diameter range of the respective plot and used to construct plot-specific diameter-dependent stand height curves using the software developed by Ehring *et al.* (1999), implemented as an option in the growth and yield database dbWW of the Forest Research Institute Baden-Württemberg (FVA). Based on these stand height curves, h_{100} was estimated, representing the height of the mean basal area tree of the 100 thickest trees ha^{-1} (d_{100}).

h_{100} is commonly used to characterise the average height of the dominant trees in a stand and is therefore a versatile indicator for a stand's potential growth. However, using plot-specific h_{100} as a database would result in a drastic reduction of the sample size (Dgl115 and Dgl114: two plots per provenance; Dgl116: 1 plot per provenance). Therefore, we included the individual data measurements of all trees of a specific plot measured for height along with the plot's characteristic h_{100}. Generally, within a stand, there is a tendency of tree height to increase with increasing diameter from suppressed to dominant trees. Therefore, we related the diameter of a specific tree within a plot to the plot's specific d_{100} as an indicator for the trees competition status and included this ratio as a linear predictor in the analyses of covariance (ANCOVA) conducted on height as the answering variable.

Statistical analyses

For assessing height growth, we carried out two analyses of covariance: The first ANCOVA examined possible differences in the height growth potential between the different sites of the experiments. Height measurements were pooled for the three experiment sites (Dgl 115 Mooswald, Dgl114 Illenberg, Dgl116 Schauinsland) and site was used as the categorical predictor. The second series of ANCOVAs addressed possible differences between the six provenances differentiated for the three sites. Here, data were pooled for provenance at the respective site and provenance used as categorical predictor. ANCOVA was executed using the glm-library of the software package STATISTICA 7.1 (StatSoft Tulsa, OK, USA) and followed by post hoc Duncan's test to investigate the levels of significance in the differences between sites (first analysis) or provenances (second analysis), respectively.

To assess differences in radial increment and tree ring $\delta^{13}C$ and $\delta^{18}O$ between provenances and sites, we applied analyses of variance followed by a Tukey-HSD post hoc test. Correlation between the isotope composition, radial increment and climatic parameters was determined by applying bivariate correlation analysis. Linear regression analysis was carried out to quantify the relations between changes in IWUE and radial increment, and between selected climatic parameters and the tree ring isotopic composition. Analysis of variance as well as correlation and regression analyses were performed by applying the respective procedures of the software package NCSS 2004 (Number Cruncher Statistical Software, Kaysville, UT, USA).

3.4 Results

Long-term height growth

The ANCOVA using the three experimental sites as categorical predictor resulted in clear differences between the experimental sites' growth potential for Douglas-fir (Table 10). The model explained the majority of variation (R^2= 0.83) and proved highly significant with an F value of 1,702. The different effects incorporated into the model were characterised as follows: constant (F = 4,277; $p < 0.0001$), linear predictor (F = 2,706; $p < 0.0001$), and experi-

ment site (F = 241, p<0.0001). The subsequent Duncan's tests revealed that the differences between the three experimental sites were statistically significant.

Table 10 Differences in the height of Douglas-fir growing for 51 years at three different experimental sites along an elevation gradient near Freiburg (Germany)
For the analysis, data obtained for the six different North American provenances, planted at each of the three experimental sites, have been pooled by site. Differences between experimental sites are significant at p<0.00001 (ANCOVA followed by Duncan's tests; categorical predictor: experimental sites, linear predictor: dbh/d100); $\bar{x}$ is the mean value, SE is standard error and N is the number of trees included

	Dgl115 Mooswald (230 m asl)		Dgl114 Illenberg (500 m asl)		Dgl116 Schauinsland (940 m asl)	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
tree height	30.6	0.23	28.2	0.17	24.8	0.45
[N]		[249]		[375]		[114]

The effects of provenance on height growth were analysed separately. At each of the three experimental sites, the provenances' height varied considerably (Table 11). The overall growth decline with site elevation (Table 10) is differentially expressed among provenances, leading to the result that some provenances show a stronger reaction to site conditions than others (Table 11). Duncan's tests following the ANCOVAs showed that a considerable number of the differences among provenances at each site proved statistically significant (Table S 4). The intensity in expressed differences was site dependent and the highest level of significance was found at the low and high elevation site.

When ranked according to height at each experimental site, only the provenance Santiam River originating from the West Cascades ranked identical at each of the three experimental sites (Table 11). This provenance invariably displayed the least height growth (rank 6). The ranking of all other provenances was more variable. Interestingly, there appeared a tendency in change of ranks along the elevation gradient of the experimental sites: with increasing elevation the provenances growing best at the low elevation site dropped in rank and vice versa (Table 11).

Table 11 Height of six different North American provenances of Douglas-fir growing for 51 years at three different experiment sites along an elevation gradient near Freiburg (Germany), (Dgl115 Mooswald 2 plots per provenance, Dgl 114 Illenberg 2 plots, Dgl 116 Schauinsland 1 plot)
The table contains mean height ($\bar{x}$) and standard error (SE) calculated from the height measurements as well as the height rank of the provenances at each of the three sites (1: tallest provenance; 6: shortest provenance)

	Dgl115 (230 m asl)		Dgl114 (500 m asl)		Dgl116 (940 m asl)	
	rank	$\bar{x}$ ± SE (N)	rank	$\bar{x}$ ± SE (N)	rank	$\bar{x}$ ± SE (N)
Duncan Paldi	1	31.5 ± 0.47 (N=40)	3	28.2 ± 0.40 (N=66)	4	24.5 ± 1.13 (N=16)
Cameron Lake	2	31.4 ± 0.48 (N=53)	2	28.4 ± 0.41 (N=76)	5	24.5 ± 1.22 (N=18)
Conrad Creek	3	31.2 ± 0.54 (N=38)	1	28.8 ± 0.39 (N=72)	3	25.2 ± 1.38 (N=17)
Timber	4	29.9 ± 0.74 (N=30)	4	28.2 ± 0.35 (N=56)	1	28.3 ± 1.13 (N=12)
Pamelia Creek	5	29.9 ± 0.65 (N=35)	5	27.7 ± 0.49 (N=45)	2	25.4 ± 1.15 (N=21)
Santiam River	6	29.6 ± 0.55 (N=53)	6	27.4 ± 0.49 (N=60)	6	22.9 ± 0.87 (N=30)

Isotopic composition of the tree rings and radial increment

There was a clear and statistically significant ($p < 0.05$) difference in the $\delta^{13}C$ between the sites Illenberg (Dgl114) and Mooswald (Dgl115) on the one hand and the high altitude site Schauinsland (Dgl116) on the other hand for the provenances Conrad Creek, Santiam River, Duncan Paldi and Timber. In these provenances, the latewood was clearly ^{13}C depleted at the uppermost compared to the two other sites (Figure 10). In the two other provenances such clear patterns could not be observed. Within the period 2002–2007, maximum year-to-year variations in $\delta^{13}C$ amounted to between 0.6 and 2.8 ‰ for the different provenances at the different sites. The strongest increase in the ^{13}C enrichment of the latewood was observed between 2002 and 2003 leading to the highest $\delta^{13}C$ values in all provenances on all sites in 2003.

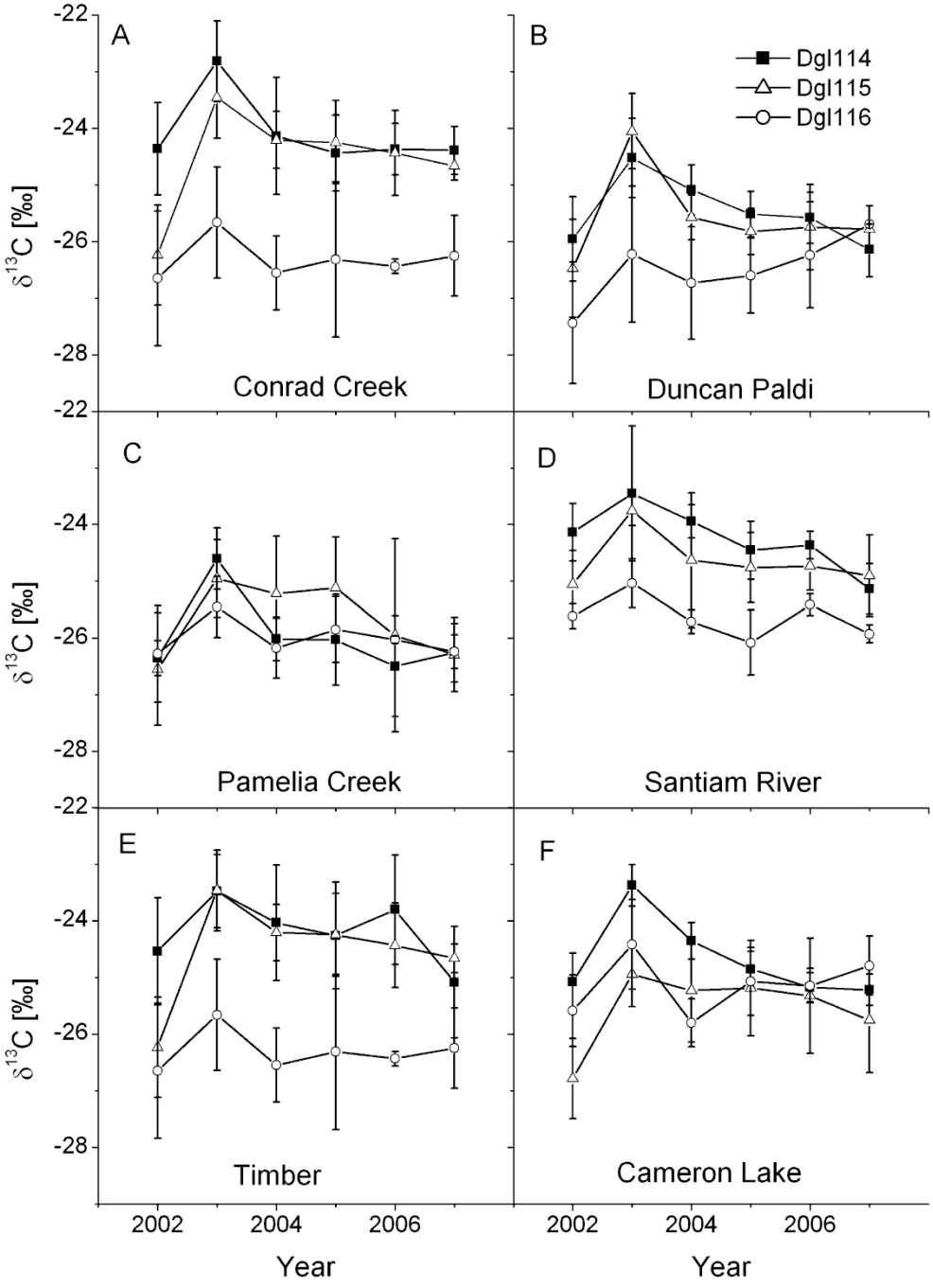

Figure 10 Carbon isotope composition ($\delta^{13}C$) in the latewood of Douglas-fir tree rings in different provenances along the altitude gradient

The figure shows the $\delta^{13}C$ in the whole wood of the provenances Conrad Creek (a), Duncan Paldi (b), Pamelia Creek (c), Santiam River (d), Timber (e) and Cameron Lake (f) from 2002 to 2007 from the experimental sites Illenberg (Dgl114; 500 m asl), Mooswald (Dgl115; 230 m asl) and Schauinsland (Dgl116; 940 m asl). Data shown are mean values ± SD (N = 4)

For $\delta^{18}O$ we found significant site differences ($p < 0.05$) for all provenances and – on average over the 7 years observed – $\delta^{18}O$ was always lowest on the high altitude site (Dgl116). Maxi-

mum year-to-year variation of $\delta^{18}O$ amounted to between 1.3 and 3.1 ‰ for the different provenances and the different sites (Figure 11). In contrast to $\delta^{13}C$ there were no temporal patterns common to all provenances and sites and the dry-hot year 2003 did not imprint a particular signal on the latewood. Only in the provenances Conrad Creek, Duncan Paldi and Cameron Lake and only on site Dgl115 (i.e. the low altitude site Mooswald), the $\delta^{18}O$ values in the latewood reached a maximum in 2003.

Analysis of variance showed no significant difference in radial growth among the provenances for the period 2002–2007 but revealed site- and year-specific differences. Radial stem growth was highest at the intermediate altitude site Illenberg (Dgl 114) and lowest at the site Dgl115 at 230 m asl (see Figure S 3). Radial increment was lowest in the years 2003 and 2006, and between 2002 and 2003 the growth decline was strongest and most pronounced on the low altitude site Dgl115.

Relation between isotopic signatures, radial increment and meteorological parameters from the actual sites of growth

We correlated the tree ring $\delta^{13}C$ and $\delta^{18}O$ values with meteorological parameters of the sites Dgl114, 115 and 116 in the years 2002–2007. The correlation matrix is shown as a heat map in Figure 12. Both, the $\delta^{13}C$ and $\delta^{18}O$ in latewood were generally negatively correlated with the amount of precipitation. In all six provenances tested, $\delta^{18}O$ was significantly correlated with the yearly sum of precipitation (P_{sum}year) and in four of six provenances with the sum of precipitation during the growing season (P_{sum}Apr–Sep). With the exception of the provenance Cameron Lake there were strong ($r < -0.7$) and significant correlations between $\delta^{13}C$ and both, P_{sum}year and P_{sum}Apr–Sep.

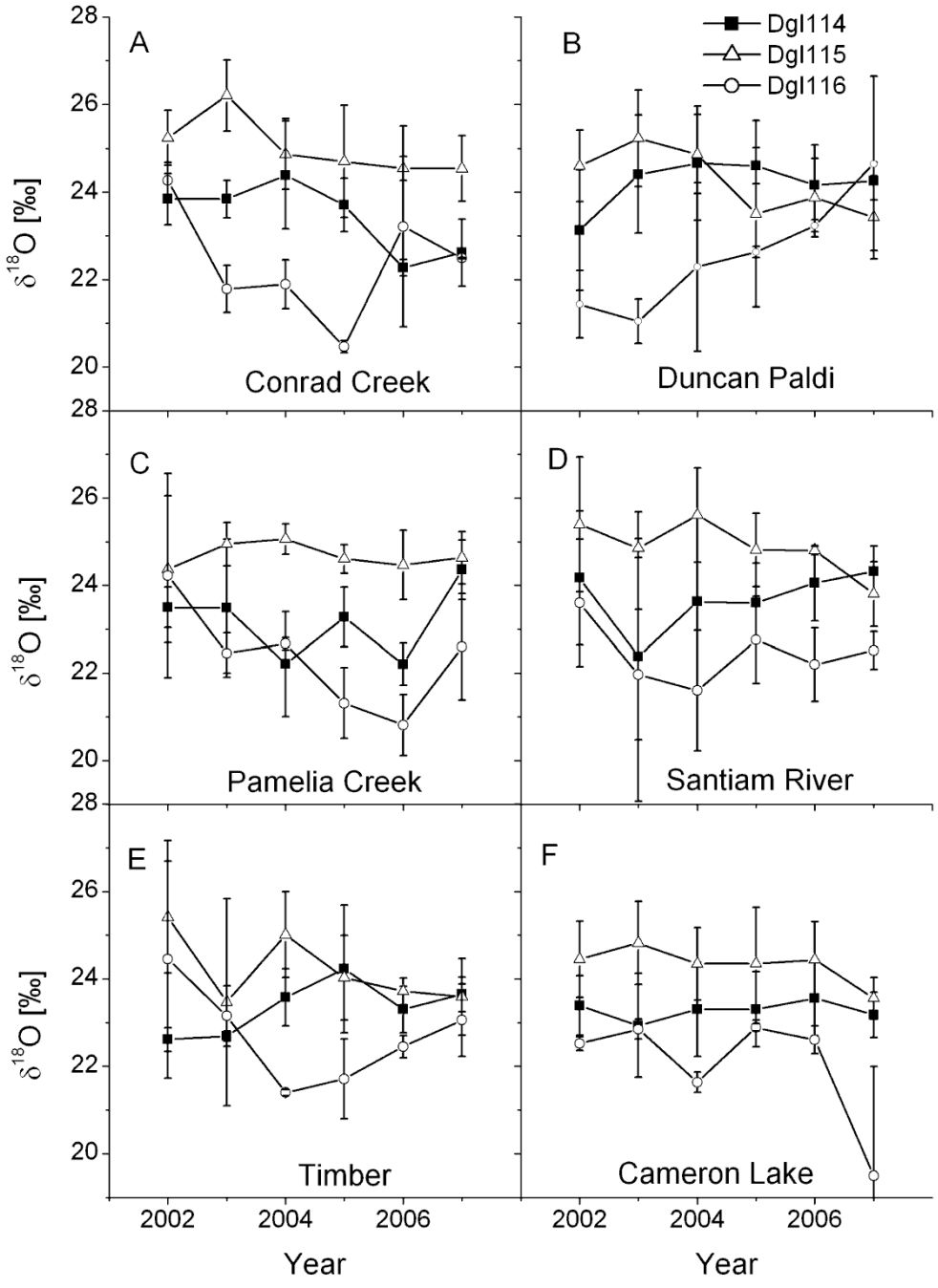

Figure 11 Oxygen isotope composition ($\delta^{18}O$) in the latewood of Douglas-fir tree rings in different provenances along the altitude gradient

The figure shows the $\delta^{18}O$ in the whole wood of the provenances Conrad Creek (a), Duncan Paldi (b), Pamelia Creek (c), Santiam River (d), Timber (e) and Cameron Lake (f) from 2002 to 2007 from the experimental sites Illenberg (Dgl114; 500 m asl), Mooswald (Dgl115; 230 m asl) and Schauinsland (116; 940 m asl). Data shown are mean values ± SD (N = 4)

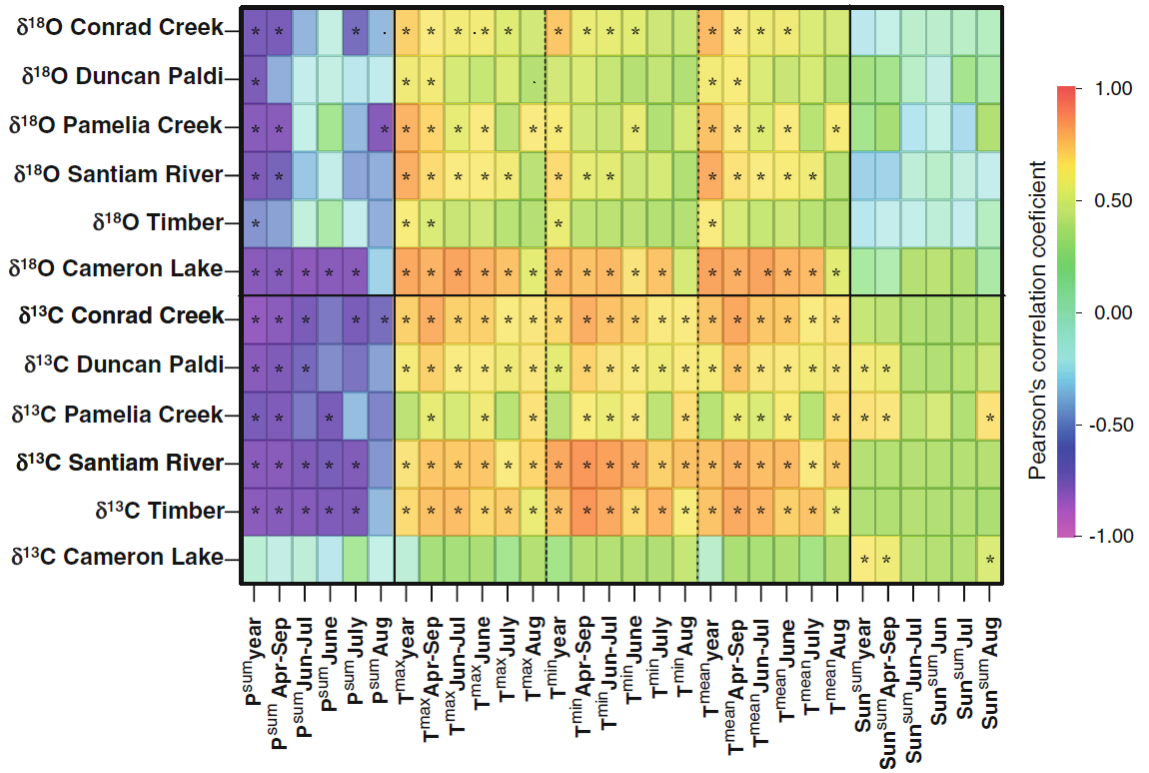

Figure 12 Correlation matrix for the oxygen and carbon isotopic signatures in the latewood of the six different Douglas-fir provenances and climatic parameters
In the correlation analysis all three sites within the altitudinal gradient as well as the years 2002–2007 where included. The colour code refers to Pearson's correlation coefficients. P_{sum} Sum of precipitation, T_{max} average daily maximum temperature, T_{min} average of daily minimum temperature, T_{mean} average of daily mean temperatures, year, April–Sep, Jun–Jul, June. July, Aug indicate the periods the climatic parameters were summed up or averaged over for a given year; *significant at $p < 0.05$

$\delta^{18}O$ in the latewood of all provenances was significantly related to daily maximum (T_{max}), minimum (T_{min}) and mean (T_{mean}) temperatures averaged over the year. For $\delta^{13}C$ the highest correlations were observed for growing season (April–September) averaged T_{max}, T_{min} and T_{mean} in the provenances Conrad Creek, Duncan Paldi, Pamelia Creek, Santiam River and Timber. $\delta^{13}C$ in tree rings of the provenance Cameron Lake was not significantly related to temperature at all. There were no or only sporadically significant correlations between sunshine hours (as a proxy for photosynthetic active radiation) and $\delta^{18}O$ or $\delta^{13}C$, respectively, in the different provenances.

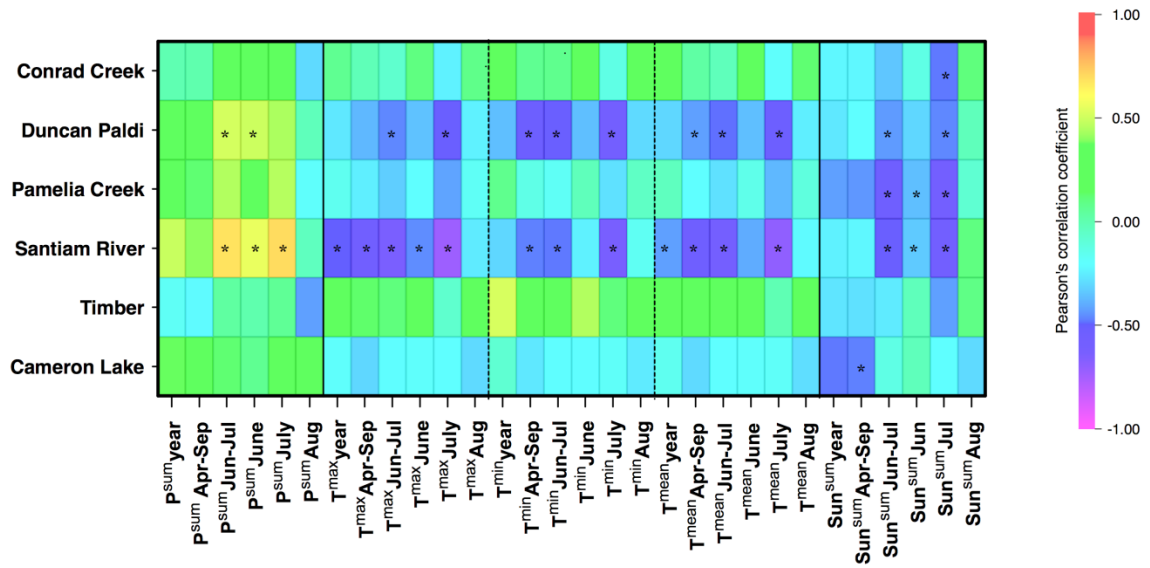

Figure 13 Correlation matrix for the radial increment of the six different Douglas-fir provenances and climatic parameters
In the correlation analysis all three sites within the altitudinal gradient as well as the years 2002–2007 where included. The colour code refers to Pearson's correlation coefficients. P_{sum} Sum of precipitation, T_{max} average daily maximum temperature, T_{min} average of daily minimum temperature, T_{mean} average of daily mean temperatures, year, April–Sep, Jun–Jul, June. July, Aug indicate the periods the climatic parameters were summed up or averaged over for a given year; *significant at $p < 0.05$

Radial increment was correlated with meteorological parameters – as varying among years and experimental sites – mainly in the provenances Duncan Paldi and Santiam River (Figure 13). For both we found positive correlation with summer precipitation and negative relation to temperature values. For all but one provenance there were negative correlations between radial growth rates and the sum of sunshine hours during various time spans of the year.

In contrast, there was no significant relation between radial growth and the isotope signatures ($\delta^{13}C$ and $\delta^{18}O$) in the latewood of the different provenances when the 6 years (2002–2007) and three experimental sites were regarded (see Figure S 4).

Regression analyses revealed that $\delta^{18}O$ in the tree ring increased with increasing T^{mean}year by approx. 0.6 ‰/°C in the provenances Conrad Creek, Pamelia Creek, Santiam River and Cameron Lake, whereas the increase was less strong (0.4 ‰/°C) for the other two provenances Duncan Paldi and Timber (Figure S 5). The $\delta^{18}O$ in the tree ring of the provenance Duncan Paldi [comment of the authors: Timber] also showed the weakest response to variations in the annual sum of rainfall (between years and sites) and decreased by 0.001 ‰/mm, whereas the slope for the other provenances ranged around -0.002 ‰/mm. Tree ring $\delta^{13}C$ response to

temperature (T_{mean}Apr–Sep) and precipitation (P_{sum}year) was also variable among provenances. Pamelia Creek responded least (0.18 ‰/°C and -2×10^{-4} ‰/mm) and Conrad Creek strongest (0.53 ‰/°C and -0.002 ‰/mm) (Figure S 5).

Isotopic composition, IWUE and growth – responses to the dry-hot year 2003

The most pronounced year-to-year difference in $\delta^{13}C$ and thus in intrinsic water-use efficiency IWUE occurred between 2002 and 2003 (Figure 10). The observed increase in IWUE was accompanied by a growth decline in some provenances, most pronounced at the low altitude site Mooswald (Dgl115). In order to explore the provenance-specific reactions towards the very dry and hot conditions in 2003 (c.f. Bréda *et al.*, 2006) more detailed, (1) $\delta^{13}C$ was plotted against $\delta^{18}O$ for 2002 and 2003 to apply the conceptual model of Scheidegger *et al.* (2000) and to detect if changes in $\delta^{13}C$ were due to changes in stomatal conductance or photosynthesis. Moreover (2) changes in $\delta^{13}C$ derived IWUE were plotted against the radial growth decline between 2002 and 2003 separated for the three sites of different altitudes.

Approach (1) revealed that in the provenances Conrad Creek, Duncan Paldi, Pamelia Creek and Cameron Lake $\delta^{18}O$ increased simultaneously with $\delta^{13}C$ between 2002 and 2003 on the low altitude site Dgl115 (Figure 14). A comparable concomitant increase between the 2 years in both, $\delta^{13}C$ and $\delta^{18}O$, was also observed on the intermediate altitude site Dgl114 (Illenberg) but only for the provenance Duncan Paldi. All other provenances on all other sites showed either a decrease in $\delta^{18}O$ with increasing $\delta^{13}C$ or no change in $\delta^{18}O$ whilst $\delta^{13}C$ increased from 2002 to 2003.

Approach (2) showed that there was a significant linear relationship between the increase in intrinsic water-use efficiency and the reduction of radial growth between 2002 and 2003 on the low altitude site Dgl115 (Figure 15). The strongest growth reduction accompanied by the greatest increase in IWUE was observed for the Conrad Creek provenance whereas Santiam River and Timber were only slightly affected. In general the growth reduction was minor on the sites Dgl114 (Illenberg) and Dgl116 (Schauinsland) as was the increase in IWUE.

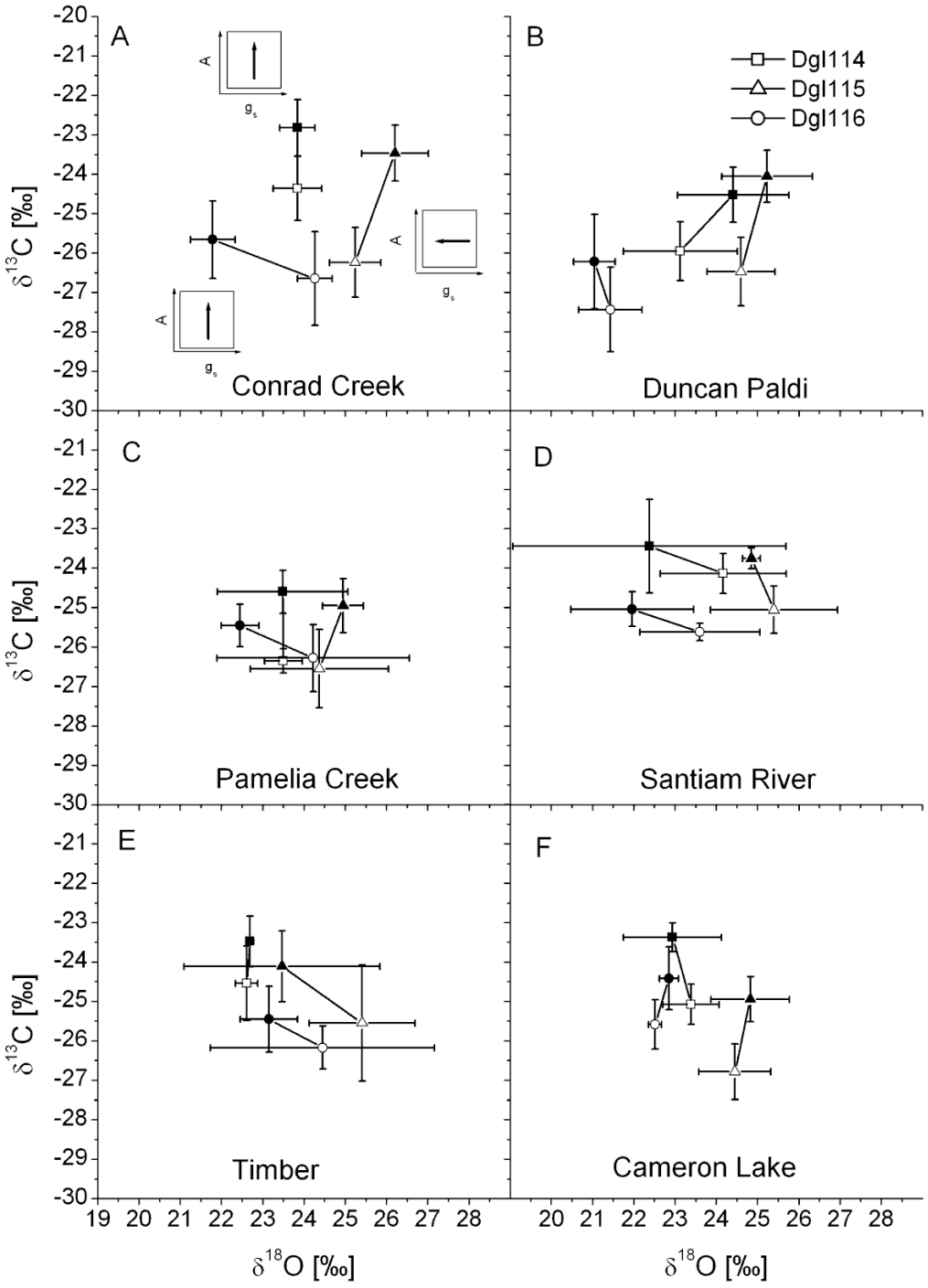

Figure 14 Concomitant change in the oxygen isotope composition ($\delta^{18}O$) and carbon isotope composition ($\delta^{13}C$) in the latewood of Douglas-fir tree rings in different provenances from 2002 to 2003

The figure shows the $\delta^{18}O$ in the whole wood of the provenances Conrad Creek (a), Duncan Paldi (b), Pamelia Creek (c), Santiam River (d), Timber (e) and Cameron Lake (f) from the experimental sites Illenberg (Dgl114; 500 m asl), Mooswald (Dgl115; 230 m asl) and Schauinsland (116; 940 m asl) plotted against $\delta^{13}C$ for 2002 (white symbol) and 2003 (black symbol). Data shown are mean values ± SD (N = 4). The insets of a indicate how the Scheidegger et al. (2000) model translates changes in $\delta^{13}C$ and $\delta^{18}O$ to changes in stomatal conductance (g_s) or photosynthetic capacity (A_n)

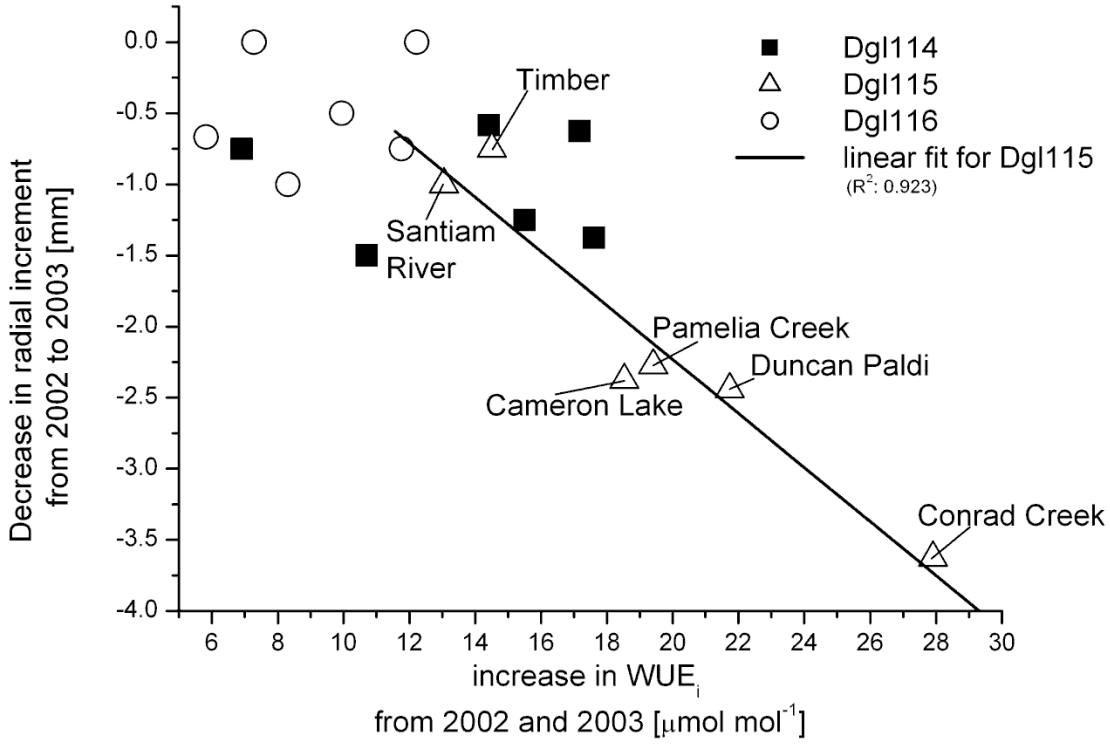

Figure 15 Change in radial increment of the Douglas-fir Provenances from the experimental sites Illenberg (Dgl114; 500 m asl), Mooswald (Dgl115; 230 m asl) and Schauinsland (Dgl116; 940 m asl) between 2002 and 2003 plotted against the change in intrinsic water-use efficiency
The provenances are indicated only for Dgl115. Data shown are mean values (N = 4)

Relation between isotopic signatures, growth and meteorological parameters from the place of origin

In order to explore if the climatic conditions at the place of origin of the different provenances are related to either the IWUE or the radial increment between 2002 and 2003 we performed correlation analyses between these parameters. There was, however, no significant correlation between the climatic parameters (long-term yearly sum of precipitation and average of temperature during the whole year or the growing season) and the IWUE increase or radial growth decrease between 2002 and 2003 (data not shown) on none of the three sites. In order to extend the examination of potential effects of the climatic conditions at the places of origin on $\delta^{13}C$, $\delta^{18}O$ to the whole 6 years time series, we averaged the isotopic composition of the latewood for the whole time period for each provenance on each site separately. Table 12 shows the correlation analysis between these parameters and the climatic variables from the place of origin. There was a significant positive correlation between $\delta^{18}O$ and the sum of precipitation

during the growing season at the place of origin and a negative correlation between $\delta^{13}C$ and mean growing season temperature, both on the low altitude site Dgl155 (Mooswald).

Table 12 Tree ring isotope composition ($\delta^{18}O$ and $\delta^{13}C$) of the different provenances separated by the experimental site and their correlation with the climatic parameters at their sites of origin
The isotopic composition ($\delta^{18}O$, $\delta^{13}C$) of each provenance on a given site (Dgl114, Dgl115, Dgl116) was averaged over the 2002-2007 period. The average values of the six provenances (N = 6) were related to the long-term average sum of precipitation of the year (MAP [abbreviations adapted for consistency; comment of the authors]) and of the growing season (MGSP) and to mean air temperature (MAT; MGST). R: Pearson's correlation coefficient

Isotopic parameters/site	Climatic parameters				
	MAP	MGSP	MAT	MGST	
$\delta^{18}O$ Dgl114	–0.62	–0.56	0.73	0.41	R
	0.185	0.244	0.097	0.417	p
$\delta^{18}O$ Dgl115	0.37	**0.84**	–0.74	–0.41	R
	0.468	0.032	0.087	0.41	p
$\delta^{18}O$ Dgl116	0.26	–0.11	0.26	–0.47	R
	0.611	0.829	0.62	0.338	p
$\delta^{13}C$ Dgl114	0.61	0.43	–0.31	–0.53	R
	0.199	0.390	0.545	0.270	p
$\delta^{13}C$ Dgl115	0.77	0.59	–0.42	**–0.82**	R
	0.072	0.220	0.405	0.049	p
$\delta^{13}C$ Dgl116	0.01	–0.02	–0.33	0.27	R
	0.986	0.973	0.528	0.597	p

3.5 Discussion

In the present study we assessed long-term performance of different Douglas-fir provenances along an altitudinal and thus precipitation and temperature gradient by studying height growth. Moreover, we characterised year-to-year responses in radial increment and carbon and oxygen isotope composition of tree ring latewood to environmental conditions with a particular focus on the dry and hot year 2003.

The significant differences in average tree height observed after 51 years clearly indicate a general decrease in height growth potential along the elevation gradient, represented by the three experiment sites. On the long term, the increase in elevation resulted in a decrease of 6 m of average height growth in Douglas-fir from the low (230 m asl) elevation sites compared to the high (940 m asl) elevation site. We assume that mainly the decrease in air tem-

perature with increasing elevation is the underlying reason for our observation. Furthermore, our data indicate that long-term height growth of the six provenances had not responded uniformly along the elevation gradient. There was only one exception: the Oregon provenance Santiam River represented at all three experimental sites the lowest provenance. Among the other five provenances there was an interesting tendency. Here, we observed a larger decrease in height growth with increasing altitude in the provenances displaying superior height growth at the low elevation site. Complementary, height growth of the two provenances representing the relatively poor ranks 4 and 5 at the low elevation site had been reduced less and had thus attained top ranks at the high elevation site.

In contrast to height growth, we did not attempt to analyse the provenances' long-term growth based on diameter (dbh, diameter at breast height) measurements obtained at the last stand measurement. As diameter growth is strongly affected among other factors by a tree's competition status, stand density, and thinning interventions, complex analytical approaches are necessary (Yue *et al.*, 2011), which would have exceeded the scope of this study. Therefore, we restricted our diameter-based analysis to annual radial increment obtained from tree ring measurements of selected sample trees for the period of the last stand measurement interval (2002–2007). As no thinning had occurred during this period, we consider it possible to neglect influences of changes in stand density dynamics on the individual trees' annual radial increment during this short period.

In contrast to the clear altitudinal patterns observed in long-term height growth of the provenances, no similar patterns were displayed in the annual radial increment observed from 2002 to 2007. Although sample trees from some provenances (Conrad Creek, Pamelia Creek, Timber) seemed to display the highest radial increment at the Dgl115 site (Mooswald) in the valley, no clear altitudinal patterns were observed in others during the observed period 2002–2007 (Figure S 3). It has to be kept in mind that the data used to analyse height and diameter growth differed significantly in magnitude with respect to temporal resolution: height growth analysis was based on 51 year totals and thus integrates environmental conditions over the entire lifespan of the trees, whereas radial increment was based on annually dissolved observations during a rather short period (2002–2007). In contrast, current tree ring widths represent only a snapshot of radial growth of already established trees to actual environmental conditions. If we focus on short-term growth response (annual radial increment) to the extremely

dry and hot year 2003, clear site-specific responses of radial increment become apparent. The increment decline between 2003 and 2005 was always smallest at the high elevation site and – with the exception of the provenance Timber – most severe at the site situated in the valley at the lowest elevation. All over Europe the very dry and hot summer 2003 caused forest ecosystems to act as carbon sources instead of sinks and resulted in strong depression of primary production (Ciais *et al.*, 2005). Various tree species strongly suffered from severe drought stress and xylem cavitation (Bréda *et al.*, 2006) and radial increment declined in coniferous and deciduous species (Pichler & Oberhuber, 2007; van der Werf *et al.*, 2007).

Dobbertin (2005) monitored basal area growth of the main tree species for 15 Swiss Level II plots and related the ratio of growth in 2003 and 2002 to plot altitude. Similar to our study the authors observed an elevation dependency of growth reduction. At altitudes below 1,200 m asl a clear growth reduction occurred in 2003 compared to 2002, whereas above 1,200 m asl stem growth increased. Meining et al. (2004) reported strong decreases in radial stem growth in 2003 for spruce sites in an altitudinal range between 510 and 1,020 m asl. For beech in Bavaria an altitude-dependent reduction of radial growth in 2003 was observed but trees growing above 800 m asl were not affected (Bayerische Landesanstalt für Wald und Forstwirtschaft, 2004). In agreement with Dobbertin (2005), we thus assume that the drought and water limitation were the limiting factors for radial growth in the dry and hot summer 2003 at low altitude. During the growing season in 2003 the modelled sum of precipitation was 289 mm at the Dgl115 site (Mooswald) in the valley and reached 397 mm at Dgl114 and 585 mm at Dgl116 (Schauinsland) at the highest elevation. As a consequence, Douglas-fir growing at higher altitude was probably less affected by water restriction. Growth was, however, still slightly reduced in 2003 at the Schauinsland site at 940 m asl. Different from Dobbertin (2005) we thus could not observe that the higher summer temperatures in 2003 favoured growth at higher altitudes.

In all provenances (except Cameron Lake) $\delta^{13}C$ in the latewood was significantly and positively correlated with growing season temperature and negatively with precipitation. As a consequence, the climatic conditions of the year 2003 imprint a clear $\delta^{13}C$ signal on the latewood of the Douglas-fir provenances at all sites (Figure 10). Comparable to growth reduction, the strongest increase in $\delta^{13}C$ was seen on the low altitude in most of the provenances (Figure 10 and 14). Since a decrease in stomatal conductance and thus decreasing c_i/c_a results in an

increase in $\delta^{13}C$ (Farquhar *et al.*, 1982), this finding supports our hypothesis of stronger water restriction at lower altitudes. However, an increase in $\delta^{13}C$ might also be a result of increased assimilation caused by higher temperatures. This temperature effect could have especially occurred at the high altitude site in 2003 where the long-term average growing season temperature is only 12.6 °C. The additional assessment of $\delta^{18}O$, as applied in the conceptual model of Scheidegger *et al.* (2000), has been regularly applied to assess if any change in $\delta^{13}C$ is due to stomatal responses or due to changes in assimilation capacity (Grams *et al.*, 2007; Gessler *et al.*, 2009b). According to the models for leaf water evaporative enrichment (Craig & Gordon, 1965; Dongmann *et al.*, 1974) refined by Farquhar and Cernusak (2005), Cuntz *et al.* (2007) and others, the water vapour pressure difference between the leaf interior and the atmosphere is a strong driver for $\Delta^{18}O$. In general, leaf stomatal conductance is strongly coupled to this pressure difference (Granier *et al.*, 2000) and thus any increase in $\Delta^{18}O$ or $\delta^{18}O$ is interpreted as a decrease in stomatal conductance g_s (Scheidegger *et al.*, 2000).

Comparable to $\delta^{13}C$, $\delta^{18}O$ showed a clear positive correlation with air temperature and a negative correlation with rainfall (Figure 12). Part of this correlation might be due to the decrease in precipitation and thus source water $\delta^{18}O$ with altitude (Poage & Chamberlain, 2001) but the difference in the altitudinal response of $\delta^{18}O$ in tree rings among provenances indicates additional provenances-specific impact of evaporative enrichment. In contrast to $\delta^{13}C$, however, the extreme year 2003 did not result in a clear $\delta^{18}O$ change in the tree ring at all sites and provenances. At the Schauinsland site, $\delta^{18}O$ decreased in most of the provenances, whereas at the Mooswald site $\delta^{18}O$ increased in four provenances (Figure 11).

We have to keep in mind that our tree ring stable isotope record covers only 6 years and as a consequence the number of degrees of freedom for the correlation analyses is rather low. Therefore, subtle differences among provenances in the relation between $\delta^{13}C$ and $\delta^{18}O$ and climatic parameters should not be over-interpreted.

When plotting the values of $\delta^{13}C$ from 2002 and 2003 against $\delta^{18}O$ for the different provenances at the different sites, it is obvious that in the provenances Conrad Creek, Duncan Paldi, Pamelia Creek and Cameron Lake $\delta^{18}O$ increases together with $\delta^{13}C$ at the low elevation site in 2003. The Scheidegger model interprets this concomitant increase in $\delta^{13}C$ and $\delta^{18}O$ as a reduction of stomatal conductance (see inserts in Figure 14a). With one exception (Duncan Paldi, Dgl114 site), $\delta^{18}O$ did not change or even decreased while $\delta^{13}C$ increased in all other

provenances or sites. Any increase in $\delta^{13}C$ without a change or accompanied by a reduction in $\delta^{18}O$ points to rather an increase in photosynthetic capacity than to a reduction in g_s (Scheidegger et al., 2000). As a consequence, the dual isotope approach indicates clear water restriction in the four provenances Conrad Creek, Duncan Paldi, Pamelia Creek and Cameron Lake but not for the two others in the valley. This finding is supported by the observed changes in intrinsic water-use efficiency between 2002 and 2003 and the concomitant radial growth reduction (Figure 15): Exactly those four provenances, which seem to reduce stomatal conductance most and thus show the strongest increase in IWUE, exhibit the strongest growth reduction at the low elevation site.

In contrast, at the higher elevation sites the Scheidegger approach suggests increased assimilation rates. However, as mentioned above, we did not observe any radial growth increase in 2003, which would be an expected consequence if increased assimilation. This apparent mismatch can be due to several reasons: (1) The increase in temperature in 2003 did not only increase the leaf level carbon assimilation but also increased respiration in all plant tissues (Ryan *et al.*, 1995) and a range of processes protecting against drought and heat stress such as the upregulation of glycolysis and the pentose phosphate pathway, which compete with growth for assimilates (Rizhsky *et al.*, 2002). (2) There are also additional factors such as transpiration rate and leaf hydraulic properties, which influence evaporative enrichment via the Péclet effect (Barbour *et al.*, 2000b) and thus might also affect and alter the output of the Scheidegger model under particular conditions (Gessler *et al.*, 2009b). The transpiration stream conveys unenriched source water to the leaf stream and dilutes the evaporatively enriched leaf water, which is referred to as the Péclet effect (c.f. Farquhar & Cernusak, 2005). Therefore, an increased transpiration in 2003 might have potentially decreased leaf water evaporative enrichment in particular provenances at the more elevated sites, where water supply might still have been sufficient.

When assessing differences in $\delta^{18}O$ in tree rings between years we have to assume that the $\delta^{18}O$ of source water the plants took up was comparable. Drought and elevated temperatures are most likely to increase $\delta^{18}O$ of the soil and thus the source water. As a consequence even comparable evaporative enrichment in 2002 and 2003 should have led to higher $\delta^{18}O$ values in the wood produced in 2003. The lower $\delta^{18}O$ in 2003 in particular provenances can thus not be explained by changes in the source water signal.

Although we identified four provenances with stomatal closure and reduced assimilation in the hot and dry year 2003, resulting in a clear decrease in stem growth at the site in the valley, there was no relation between this drought sensitivity and the environmental conditions at the site of origin. From this observation we conclude that long-term climatic conditions at the locations of origin averaged over the year or the growing season as used for our correlation analysis might be poor indicators for the occurrence of drought stress. The averaged data do not provide information on month-to-month and year-to-year variability of precipitation and thus are not indicative for the occurrence of regular or sporadic intensive drought events and consequently for the potential adaptation of provenances to such conditions. On the other hand, the positive correlation between $\delta^{18}O$ and average precipitation at the location of origin and the negative correlation for $\delta^{13}C$ and temperature across all provenances grown at the valley site might indicate at least some general predictive potential of the original average climate conditions for the physiological performance. The combined oxygen and carbon isotopic data and their opposite correlation with temperature and precipitation (Table 12) point to the fact that under the average conditions at the valley site during the 6-years period from 2002 to 2007 the stomata are likely to be more open (lower $\delta^{13}C$ and $\delta^{18}O$) in provenances originating from warmer and drier regions compared to those from cooler and wetter regions (higher $\delta^{13}C$ and $\delta^{18}O$).

Interestingly, the two provenances displaying the least height growth potential at the low elevation site (Timber, Santiam River) appear to display overall the least response to the hot and dry conditions in 2003 at this site. It remains to be studied if this reflects genotypic adaptation to dry environments and whether this is caused e.g. by an increased investment of these provenances into belowground biomass and fine root production during their lifetime. This would be an adaptive mechanism providing efficient exploitation of soil water resources in dry periods (Meier & Leuschner, 2008).

In conclusion, different Douglas-fir provenances clearly vary in their drought sensitivity as shown by the radial growth decline in the extreme dry and hot year 2003. The growth decline in the provenances Pamelia Creek, Cameron Lake, Duncan Paldi and Conrad Creek was clearly attributed to decreased stomatal conductance but was not related to the long-term average climate at the places of origin of the provenances. The two provenances, which were least affected by the extreme drought event, showed the lowest long-term height growth potential

at the driest site. With the provenances tested here, we thus see a trade-off between long-term growth performance under 'average' conditions and the response to extreme events, which certainly needs further examination for example by assessing long-term tree ring series and physiological mechanisms such as carbon allocation (e.g. Sala *et al.*, 2012). This trade-off needs to be taken into account for a sustainable and economically successful cultivation of Douglas-fir and thus site-specific evaluations of the probability of extreme drought events in future will help to select the appropriate provenances.

3.6 Acknowledgments

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3.7 Supplementary Material

Table S 4 Statistical significance of differences between the height of six different North American provenances of Douglas-fir growing for 51 years at three different experiment sites along an elevation gradient near Freiburg (Germany); (Mooswald : 2 plots per provenance, Illenberg: 2 plots, Schauinsland: 1 plot)
The table summarizes the results of Duncans' tests following ANCOVAs employing provenance as categorical predictor and the ratio dbh/d100 as linear predictor. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; n.s.: not significant

	Duncan Paldi	Conrad Creek	Pamelia Creek	Santiam River	Timber
Mooswald (230 m asl) *ANCOVA: a) overall model R^2=0.81, F=178, p<0.0001 b) parameters: constant (F=2724; p<0.0001), linear predictor (F=2588, p<0.0001), provenance (F=11, p<0.0001)*					
Cameron Lake	n.s.	n.s.	****	****	****
Duncan Paldi		n.s.	****	****	****
Conrad Creek			***	****	***
Pamelia Creek				n.s.	n.s.
Santiam River					n.s.
Illenberg (500 m asl) *ANCOVA: a) overall model R^2=0.76, F=198, p<0.0001 b) parameters: constant (F=1626; p<0.0001), linear predictor (F=1160, p<0.0001), provenance (F=8, p<0.0001)*					
Cameron Lake	n.s.	n.s.	*	**	n.s.
Duncan Paldi		n.s.	n.s.	**	n.s.
Conrad Creek			***	****	n.s.
Pamelia Creek				n.s.	n.s.
Santiam River					**
Schauinsland (940 m asl) *ANCOVA: a) overall model R^2=0.93, F=256, p<0.0001 b) parameters: constant (F=1005; p<0.0001), linear predictor (F=1388, p<0.0001), provenance (F=5, p=0.0002)*					
Cameron Lake	n.s.	n.s.	n.s.	***	****
Duncan Paldi		n.s.	n.s.	***	****
Conrad Creek			n.s.	****	****
Pamelia Creek				****	****
Santiam River					****

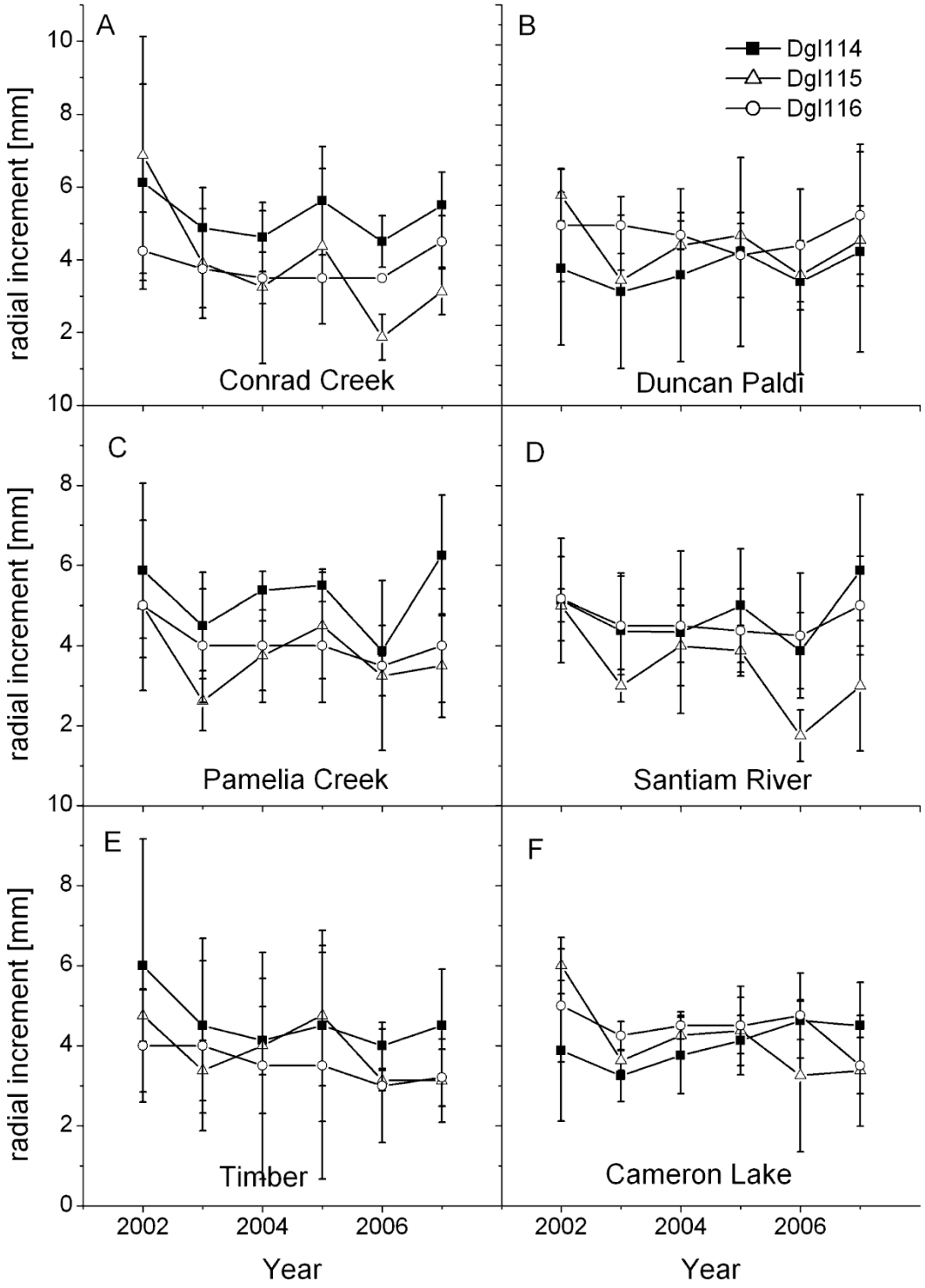

Figure S 3 Radial growth of different Douglas-fir provenances along the altitude gradient
The figure shows tree ring width at breast height of the provenances Conrad Creek (A), Duncan Paldi (B), Pamelia Creek (C), Santiam River (D), Timber (E) and Cameron Lake (F) from 2002 to 2007 from the experimental sites Illenberg (Dgl114; 500 m asl), Mooswald (Dgl115; 230 m asl) and Schauinsland (116; 940 m asl). Data shown are mean values ± SD (N=4)

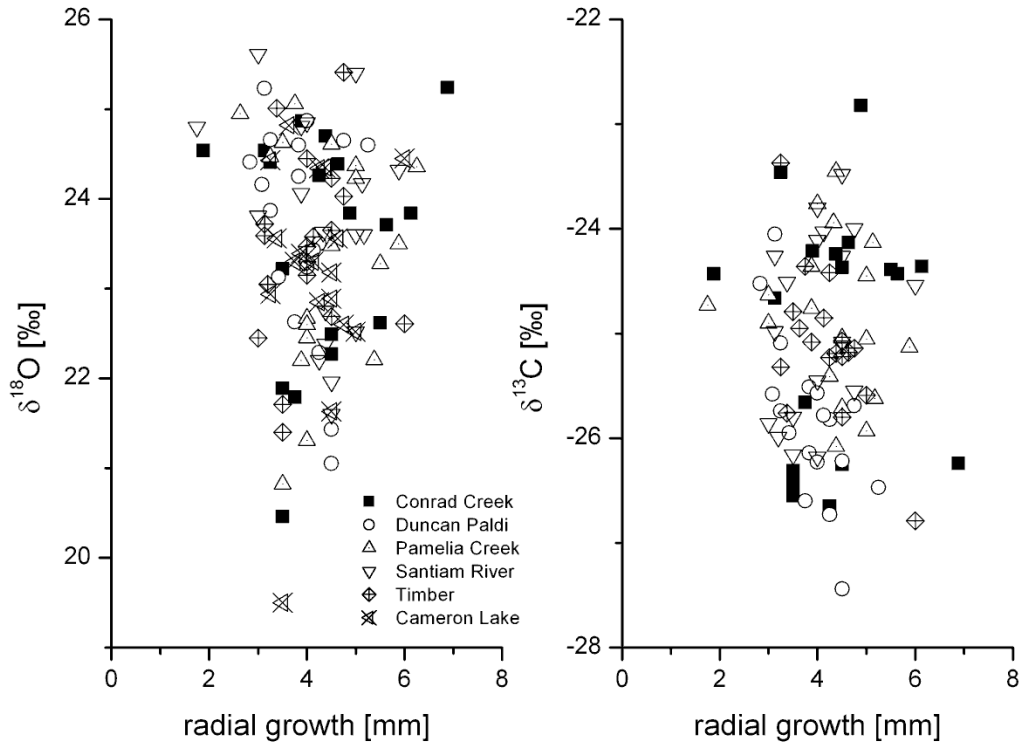

Figure S 4 Oxygen ($\delta^{18}O$) and carbon isotopic composition ($\delta^{13}C$) in the latewood of the different Douglas-fir provenances plotted against radial increment
Data shown are mean values (N = 4) from 6 years and from 3 sites along an altitudinal gradient

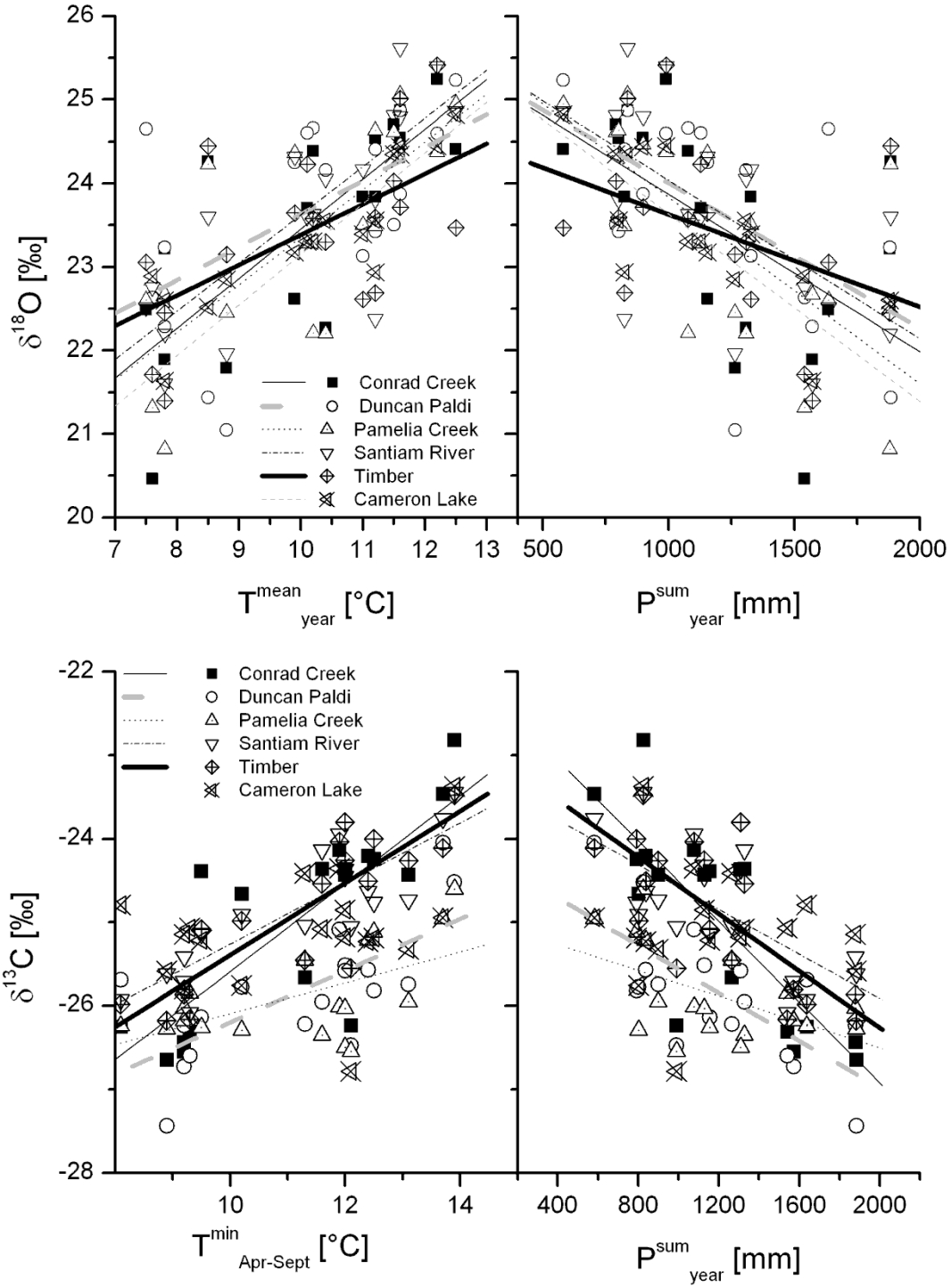

Figure S 5 Regression analyses for carbon and oxygen isotopes signatures isotopic signatures in the latewood of the 6 different Douglas-fir provenances and climatic parameters from the actual sites of growth in south-western Germany

4 Response of Douglas-Fir Seedlings to a Simulated Heat Wave

Title of the publication: Douglas-Fir Seedlings Exhibit Metabolic Responses to Increased Temperature and Atmospheric Drought

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4.1 Abstract

In the future, periods of strongly increased temperature in concert with drought (heat waves) will have potentially detrimental effects on trees and forests in Central Europe. Norway spruce might be at risk in the future climate of Central Europe. However, Douglas-fir is often discussed as an alternative for the drought and heat sensitive Norway spruce, because some provenances are considered to be well adapted to drier and warmer conditions.

In this study, we identified the physiological and growth responses of seedlings from two different Douglas-fir provenances to increased temperature and atmospheric drought during a period of 92 days. We analysed (i) plant biomass, (ii) carbon stable isotope composition as an indicator for time integrated intrinsic water-use efficiency, (iii) apparent respiratory carbon isotope fractionation as well as (iv) the profile of polar low molecular metabolites.

Plant biomass was only slightly affected by increased temperatures and atmospheric drought but the more negative apparent respiratory fractionation indicated a temperature-dependent decrease in the commitment of substrate to the tricarboxylic acid cycle. The metabolite profile

revealed that the simulated heat wave induced a switch in stress protecting compounds from proline to polyols.

We conclude that metabolic acclimation successfully contributes to maintain functioning and physiological activity in seedlings of both Douglas-fir provenances under conditions that are expected during heat waves i.e. elevated temperatures and atmospheric drought. Douglas-fir might be a potentially important tree species for forestry in Central Europe under changing climatic conditions.

4.2 Introduction

Global climate change is expected to affect forest growth and productivity (IPCC, 2007, 2013). The projected increase in air temperature is the most direct and best predictable effect of increasing tropospheric CO2 concentrations (Smith *et al.*, 2007). In addition to a continuous increase in air temperature, the frequency of climate extremes, including heat waves, has been predicted to strongly increase (Easterling *et al.*, 2000; Meehl & Tebaldi, 2004; Schär *et al.*, 2004; De Boeck *et al.*, 2010; IPCC, 2013). Tree growth and physiological performance will depend on both the acclimation to increased temperatures and the resistance against extreme events such as the hot-dry summer 2003 in Central Europe (Bréda *et al.*, 2006). Our insights into the adaptation and acclimation potential of tree species towards climatic change are still incomplete and especially the effects of heat waves i.e. periods of increased temperature in concert with drought (Ciais *et al.*, 2005; Bréda *et al.*, 2006; Allen *et al.*, 2010) on the physiology of trees are not well understood (e.g. Rennenberg et al. 2006).

Compared to deciduous tree species, evergreen species show a weak growth increase (Way and Oren 2010) or no growth response to constant, yet, moderate temperature increases (Carter 1996). In Douglas-fir, the 2003 drought and heat wave showed diverging effects on radial growth ranging from no response at moist sites to significant decreases at dry sites in Germany and Switzerland (Jansen *et al.*, 2013; Lévesque *et al.*, 2014). A species' response to increased temperature depends on its thermal optimum for photosynthesis and growth, and its current operation either at or below the optimum (Way & Oren, 2010). Rising temperatures may stimulate assimilation, physiological performance, and growth below the thermal opti-

mum, but may lead to reduced performance above species- or provenance-specific thresholds (Rehfeldt *et al.*, 2002; Ryan, 2010).

Changes in temperature might not only affect photosynthesis and thus carbon supply for growth but also sink activity (Koerner, 2003) and, as an underlying mechanism, the allocation of assimilated C to different metabolic pathways (Kaplan *et al.*, 2004). Shifts in the commitment of metabolic pathways can be detected by changes in the apparent respiratory carbon isotope fractionation (Werner & Gessler, 2011). Leaf respired CO_2 is generally ^{13}C-enriched compared to the putative respiratory substrate (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Werner & Gessler, 2011), mainly due to fragmentation of the substrate molecule (fragmentation fractionation) (Tcherkez et al. 2003 and 2004) and potentially due to enzyme related isotope effects (Werner *et al.*, 2011; Werner & Gessler, 2011). During glycolysis, the C-1 of pyruvate derived from ^{13}C-enriched C-3 and C-4 of glucose is decarboxylated by pyruvate dehydrogenase (PDH) and, consequently, the PDH reaction releases ^{13}C-enriched CO_2 (Tcherkez *et al.*, 2003). The remaining part of the pyruvate molecule can enter the tricarboxylic acid (TCA) cycle during which relatively depleted CO_2 is released. Alternatively, the remaining part of pyruvate might be used for the production of acetogenic lipids and various secondary metabolites (cf. Werner and Gessler 2011). Assuming that the whole pyruvate molecule were oxidised in the TCA cycle, the $\delta^{13}C$ of CO_2 released from respiration would equal that of the glucose substrate. The more pyruvate that is committed to metabolic pathways other than the TCA cycle, the higher the contribution of the PDH-released ^{13}C enriched CO_2 to the total respiratory CO_2 emission, and the more negative the apparent respiratory fractionation.

Moreover, metabolite profiling provides information about the accumulation of precursors of secondary metabolites or stress related compounds (Kaplan *et al.*, 2004). For instance, proline accumulation is associated with various environmental stresses (see e.g. Stewart et al. 1980; Taylor 1996), but can also be toxic to cells (Hellmann et al. 2000; Mani et al. 2002), especially under heat stress (Rizhsky et al. 2004). Alternative osmolytes such as polyols (Hoekstra *et al.*, 2001; Taji *et al.*, 2002) might be crucial for maintaining leaf turgor under conditions of drought and high temperatures. Especially the polyol D-pinitol is known to be present in large amounts in conifers (e.g. Riikonen et al. 2012) and might play an important role in osmotic adjustment in these plants. Moreover, heat stress is known to generally upregulate metabolites

related to the shikimate pathway (Kaplan *et al.*, 2004), which is related to the synthesis of cell wall components, lignin, aromatic amino acids, phenylpropanoids and flavonoids, and is also closely linked with carbohydrate metabolism (Buchanan *et al.*, 2000).

In Central Europe, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is often discussed as a potential alternative for the economically important Norway spruce (*Picea abies* (L.) Karst.), the performance of which could be seriously threatened by climate change (Hanewinkel *et al.*, 2010). Douglas-fir is a remarkably productive species with a high economic potential and it is assumed to display a comparably high resistance against drought and heat (Brandl, 1989; Heidingsfelder & Knoke, 2004). Within an extended natural range, Douglas-fir populations grow under various climatic conditions and these different environmental drivers may act as selective force on the genetic and physiological growth response of different provenances (Bradley St Clair & Howe, 2007; Sork *et al.*, 2013). For instance, our current understanding of water relations in Douglas-fir seedlings suggests that populations from regions with relatively cool winters and arid summers are potentially best adapted to warm and dry conditions (Bansal *et al.*, 2014). With climate change, future summer temperatures in Central Europe will probably more frequently exceed the thermal optimum for photosynthesis in Douglas-fir (10-20 °C, Doehlert and Walker 1981; Lewis et al. 2001). Heat waves will be most likely accompanied by reduced precipitation and high water vapour pressure deficit (VPD) resulting in atmospheric drought (De Boeck *et al.*, 2010). This calls for a better understanding of the interactive impact of heat and atmospheric drought on Douglas-fir physiology and growth in order to inform forest managers about the potential of this species. One issue that is of major importance is the impact of heat and drought on tree seedlings as they represent the future of a forest stand and there are indications that the acclimation to environmental stresses strongly depends on ontogeny (Cavender-Bares & Bazzaz, 2000) and that seedlings are more intensively affected by environmental stressors compared to adult individuals (Gessler *et al.*, 2004).

The aim of this study was to identify seedling physiological and growth responses of Douglas-fir from two different provenances to a simulated heat wave accompanied by atmospheric drought. We examined the two provenances Monte Creek (MC) and Pend Oreille (PO), originating from the *menziesii-glauca* transition zone in southern interior British Columbia and the *glauca* zone in Northwest Washington State, respectively (Table 13; cf. Little 1971; Sorensen

1979; Randall and Berrang 2002; Kohnle et al. 2012). These provenances are originating from the rather dry part of the natural range of Douglas-fir, in comparison to the coastal areas of Washington and British Columbia. The summer air temperatures are comparable for the two provenances, however, the MC provenance region receives only approx. half the amount of rainfall compared to the PO region and thus individuals from the MC provenance might be better adapted to heat accompanied by atmospheric drought.

To examine effects of heat waves on physiology and growth of Douglas-fir, seedlings were grown under high temperature (control +10°C) combined with high vapour pressure deficit (control +1.6 kPa) as well as under control conditions (20°C and 0.35 kPa) for more than three months (Table S 5). The 2003 heat wave showed whole July temperature anomalies (compared to 2001) of up to +10°C for large areas of Central Europe (Stockli & Simmon, 2003) indicating the realistic temperature range of our treatment. We analysed plant biomass, carbon stable isotope composition as an indicator for time integrated intrinsic water-use efficiency (IWUE = assimilation/stomatal conductance; Farquhar et al. 1989), apparent respiratory fractionation in order to assess temperature-dependent changes in the commitment of substrate to the TCA cycle (Priault et al., 2009; Werner et al., 2011), as well as the profile of polar low molecular metabolites.

We hypothesize that (1) the increased temperature together with atmospheric drought (i.e. elevated VPD) will reveal metabolic plasticity where precursors are fed into alternative metabolic pathways resulting in altered metabolite profiles. We further assume (2) that heat and atmospheric drought during three months lead to reduced biomass production and changes in biomass allocation patterns. Finally we hypothesize (3) that the MC provenance is less affected by the combined heat and atmospheric drought impact as a consequence of its potential adaptation to the lower water availability at the area of origin.

4.3 Materials and Methods

Plant material and growing conditions

Ethics statement: We did not perform field work but worked with seedlings under controlled conditions in climate chambers. The study did not involve endangered or protected species.

Douglas-fir seedlings were obtained from forest tree nurseries (Nursery Services Interior, Vernon, Canada and Webster Forest Nursery, Olympia, WS, USA) and no specific permission was required for buying the seedlings. Import to Germany and growing plants under quarantine regulations was permitted by the Regierungspräsidium Freiburg; Pflanzengesundheitsdienst, Freiburg, Germany.

Table 13 Provenances, geographic location and climatic conditions in the regions of origin
Climatic parameters as mean annual temperature (MAT), mean annual precipitation (MAP), mean summer (May-Sep) temperature (MST) and precipitation (MSP), Hargreaves climatic moisture deficit (CMD, sum of the monthly difference between a reference evaporation and precipitation) is calculated with Climate WNA as described in Wang et al. (2012)

Name	Variety	Elevation [m asl]	Latitude N	Longitude W	MAT [ºC]	MAP [mm]	MST [ºC]	MSP [mm]	CMD [mm]
Canada, Southern Interior British Columbia, Thompson Okanogan									
Monte Creek	interior /transition	850±50	50.7	-120	5.2	362.0	13.56	171.0	433.0
USA, Northeast Washington State, Okanogan Highlands									
Pend Oreille	interior	925±75	48.3±0.7	-117.7±0.7	6.5	735.7	14.78	219.5	396.7

Three to four years old seedlings of the Douglas-fir provenances Monte Creek (MC) and Pend Oreille (PO) (see Table 13) were grown in environmental chambers in 4L pots with substrate consisting of commercial potting soil (1 part Anzucht- und Pikiererde, Ökohum, Herbertingen, Germany; 1 part perlite; 1 part sand). The substrate was supplied with long-term fertilizer (Osmocote Exact high-end 5-6, 15N+9P+12K+2Mg, 3g L^{-1} substrate, The Scotts Company, LLC, Marysville, OH, USA / ICL, Tel Aviv, Israel). The chambers were illuminated by sodium-vapour lamps (NC 1000-00/-01/-62, Narva, Plauen, Germany, with 6, 8 and 10-11% red light, respectively).

Over a period of 78 days, plants were stepwise acclimatized to control conditions (Table S 5). The following treatment phase covered 92 days and started after full leaf development. The aim of this approach was to simulate a heat wave comparable to the one in Europe in 2003, which occurred in mid summer after foliage had been fully developed. During the first 10 days of acclimation, the temperature was raised from 10/10°C (light/dark period) to 20/15°C. Plants in the treatment group were exposed to 25/20°C for three days before the 30/25°C treatment started. Coniferous needle leaves are strongly coupled to the environment (Barbour

et al., 2002), resulting in leaf temperature equalling air temperature. During the acclimation period, the photoperiod was extended from 12h to 16h and the light intensity was raised from 250 to 600 µmol m^{-2} s^{-1} PPFD to be above the light saturation point of Douglas-fir (Lewis *et al.*, 2000).

During the treatment phase, VPD was held at 0.35 kPa (in the light period) in the control and 1.91 kPa in the treatment, the latter assumed to represent atmospheric drought. In both groups, soil water content was kept at optimum levels (approx. at field capacity). During the time of experimentation, the plants had three needle age classes developed at the beginning of the current year 2010 (N10), in the previous year (N09), and in the year 2008 (N08).

Climate data for the region of origin from the two provenances examined was obtained with the software ClimateWNA by downscaling PRISM monthly data (Daly *et al.*, 2002) for the reference period (1961-1990), and calculating seasonal and annual climate variables for the specific locations based on latitude, longitude, and elevation (s. Table 13) as described in Wang et al. (2012). For the MC provenance, the geographic location as provided by the seedling supplier (Nursery Services Interior, Vernon, BC, Canada) was used as input parameter. The PO provenance originates from the seed zone Pend Oreille described by Randall and Berrang (2002). A grid of geographic locations within the seed zone was used as input for ClimateWNA and the resulting multiple climate data were averaged. Annual precipitation sum (mm), mean summer precipitation sum (mm) and mean annual temperature (°C) in the PO zone of origin are around 374 mm, 48 mm and is 1.26°C higher than in the MC zone of origin (Table 13).

Experimental design and biomass sampling

We conducted repetitive sampling during a period of 92 days after treatment start (DAT), including measurements during a diel course and a complete harvest of the plants at the end of the experimental period. On DAT 1 and 41 we sampled current-year needles of 20 plants (N=5) for carbon isotope analysis. We conducted a full diel course on DAT 64 with 12 plants (N=3) and six sampling time points (four during the light and two during the dark period) to study the carbon isotopic composition of respired CO_2 and of potential organic sources for respiration, as well as respiration rates. At each time point (2, 6, 10, 14, 18, and 22 h ±2 h), we sampled current-year needles, fine roots, and respired CO_2 from canopy chambers (see

below). Needle and root samples were taken from separate plants to avoid interferences with the CO_2 samples. At the end of the experimental period, from day 76 to 92 after treatment start, 36 plants were completely harvested for biomass, isotope and metabolite analyses, and separated into needles (subdivided into 3 age classes), twigs, stems (subdivided into bark and wood), fine and coarse roots, and measured for fresh weight. Subsamples were dried at 105°C for 24 h for assessing dry weight while the rest of the samples was immediately frozen in liquid nitrogen and stored in -80°C until further analyses.

Stable isotope analyses of organic matter and CO_2

$\delta^{13}C$ in organic matter was determined in the water-soluble fraction (WSOM; water-soluble organic matter) of needles according to Gessler et al. (2009) and Ruehr et al. (2009). 250µl of the extracts were dried under vacuum and stored in a desiccator. The isotopic composition of WSOM is an excellent proxy for sugars and thus recent assimilates (Gessler *et al.*, 2009c). In addition, $\delta^{13}C$ was determined in the total organic matter fraction (bulk) of several plant compartments, i.e. in the dried and homogenised plant material.

For $\delta^{13}C$ analysis, approx. 0.3 mg of the dried homogenised total organic matter samples and the dried WSOM extracts samples were combusted in an Flash HT elemental analyzer (ThermoFinnigan, Bremen, Germany) coupled via a Conflo III interface to an isotope ratio mass spectrometer (Delta V Advantage, ThermoFisher, Bremen, Germany). Carbon isotopic values were expressed in δ notation relative to the Vienna Pee Dee Belemnite (VPDB). The precision for measurements as determined by repeated measurements of standards (n = 10) was better than 0.10 ‰.

For the assessment of $\delta^{13}C$ in canopy respired CO_2 ($\delta^{13}C_R$), we installed Perspex® respiration chambers around the upper part of the canopy (5.39L volume, custom-made production, Herbert Geißler GmbH & Co. KG, Freudenberg, Germany) according to Kodama et al. (2011). The chambers enclosed the needles and twigs from the approx. upper half of the crown and were supplied with interior fans to ensure mixing of air, sealed with an inert plastic material (Terostat VII®, Henkel Teroson GmbH, Heidelberg, Germany), completely darkened with aluminium tape to prevent photosynthesis, and provided with air-tight septa to allow gas sampling with hypodermic needles and syringes. The gas samples were taken 0, 1, 5, 12 and 20 min after closing the chambers and subsequently transferred into pre-evacuated exetainers

(12 ml, Labco Ltd, High Wycombe, UK). The chambers were removed directly after each sampling phase.

Gas samples were analysed for δ $\delta^{13}C$ and [CO_2] in an isotope ratio mass spectrometer (Delta V Advantage, ThermoFisher, Bremen, Germany) coupled to a gas bench as described by Kodama et al. (2008). $\delta^{13}C_R$ was determined with mixing models using the Keeling plot approach (Keeling, 1958, 1961) and the Miller-Tans approach (Miller & Tans, 2003). The [CO_2] range can impact the outcome of mixing models (Kayler et al. 2010). In our case, both approaches resulted in very similar results and we thus applied an average for $\delta^{13}C_R$. Respiration rates were calculated from the linear increase in CO_2 concentration over time and based on the total leaf, twig, and stem area. Apparent respiratory fractionation ($a\Delta_R$) was calculated as the difference between $\delta^{13}C$ of current-year needle WSOM and $\delta^{13}C$ of respired CO_2 according to Gessler et al. (2009).

Analysis of metabolites by gas chromatography–mass spectrometry (GC-MS)

Polar low-molecular-weight metabolites were extracted from N09 and N10 needles and derivatised according to a modified method from Kreuzwieser et al. (2009) and Erxleben et al. (2012). For each sample, approximately 50 mg of homogenized frozen tissue powder was weighed into a pre-frozen 2 mL round-bottom Eppendorf tube and 500 µL of cold 85% (v/v) methanol (Sigma) were added as extraction medium. 1 µg ml-1 ribitol was used as internal standard. Tubes were rapidly heated to 65°C and shaken at 1,400 RPM for 15 min after brief vortex. 50 µL aliquots of supernatant were dried under vacuum in 1.5 ml microfuge tubes after centrifugation. Dried extracts were methoximated by adding 20 µL of a 20 mg mL-1 solution of methoxyamine hydrochloride in anhydrous pyridine (Sigma) and incubated at 30°C for 90 min with shaking at 1400 RPM. For trimethylsilylation, 70 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma) was transferred to each tube and incubated at 37°C for 30 min with 1,400 RPM shaking. 10 µL of an n-alkane retention index calibration mixture [n-Alkane- Mix 16 (C10-C40 even), Cat.-No.:14640, concentration: 50 µg ml-1 in n-hexane; Neochema, Germany] was then added to each sample. After short vortex, reaction mixtures were centrifuged at 14,000 g, 20°C for 2 min and then 80 µL of supernatant were transferred to amber GC-MS vials with low volume inserts and screw top seals (Agilent Technologies, Palo Alto, CA, USA) for GC-MS analysis.

Derivatised metabolite samples were analysed on an Agilent GC/MSD system comprised of an Agilent GC 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with a GERSTEL MultiPurpose Sampler (MPS2-XL, GERSTEL, Mülheim, Germany) and 5975C Inert XL EI/CI MSD quadrupole MS detector (Agilent Technologies, Palo Alto, CA, USA). The capillary column used was HP-5MS 5% Phenyl Methyl Silox, length: 30 m, diameter: 0.25 mm, film thickness: 0.25 µm (Agilent Technologies, Palo Alto, CA, USA). GC-MS run conditions were set up according to Erxleben et al. (2012) with some slight adaptations. The GC column oven was held at the initial temperature of 80°C for 2 min and then to 325°C at 5°C min-1 before being held at 325°C for 10 min. Total run time was 61 min. Transfer line temperature was 280°C. MS source temperature was 230°C.

The raw data files were processed with the free AMDIS (automated mass spectral deconvolution and identification system, version 2.69) software supplied by NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA). Mass spectra were searched against a user-defined metabolite database based on the Golm Metabolome Database (Kopka *et al.*, 2005) and identified based on retention index and spectrum similarity match. A relative quantification of metabolite peaks was done by calculating the areas of the extracted ion chromatograms. The area value for each metabolite was standardised by sample weight, then normalized for the area measured for the internal ribitol standard, and corrected for blank values where only solvents and derivatisation reagents were measured. Metabolites detected in less than 50% of all the replicates of each provenance and needle age class were discarded for the comparison between treatments and controls. Treatment-specific differences of the corrected and normalised areas are given as log ratios of treatments vs controls.

Statistical analyses

All measured parameters were characterized by descriptive statistics (means and standard deviations of the means). Statistical analyses were carried out with R® 3.0.2 (R Development Core Team 2013). Normality of residuals was tested with the Shapiro-Wilk test and was rejected when p-values were smaller than 0.1. Homogeneity of variances was tested with the Fligner-Killeen test on grouped data and rejected when p-values were smaller than 0.05. A 2-way analysis of variance was conducted to assess the effects of provenance, treatment and their interactions. In case of non-normality of residuals, ANOVA was performed on ranks. In

case of interacting effects in the 2-way ANOVA, a 1-way ANOVA was conducted for single parameters in order to determine effects separately. Tukey's post-hoc was used following the ANOVA analysis for multiple comparisons among the groups.

4.4 Results

Plant biomass

Treatment-specific effects on the dry weight of Douglas-fir seedlings were only observed for needles of the oldest age class N08 (Figure S 6A). The biomass of these needles decreased significantly under high temperature and VPD. All other needle age classes and tissues as well as total biomass were not significantly affected by high temperature and atmospheric drought treatment.

There was no significant temperature effect on the ratio between above- and below-ground biomass, indicating that above- to below-ground biomass allocation was not affected by the treatment (Figure S 6B). However, the ratio between fine-root and coarse-root biomass was significantly decreased, whereas the ratio between current-year needles and fine roots was increased.

Carbon isotope composition of plant organic matter

In both provenances and in both treatments, $\delta^{13}C$ in WSOM of current-year needles (N10) decreased during the treatment period by up to 1.9‰ for MC and 2.0‰ for PO (Figure 16). $\delta^{13}C$ in WSOM of all analysed needle age classes was significantly affected by provenance at the end of the treatment period ($p<0.005$ for N10) with lower values for PO. The heat and high VPD treatment did not affect $\delta^{13}C$ of WSOM in the three needle age classes (Figure 16). Significant effects of high temperature and VPD on $\delta^{13}C$ were found only in fine root WSOM of PO (Figure 16). Bulk total organic matter $\delta^{13}C$ in coarse roots and bark were also not affected by treatment.

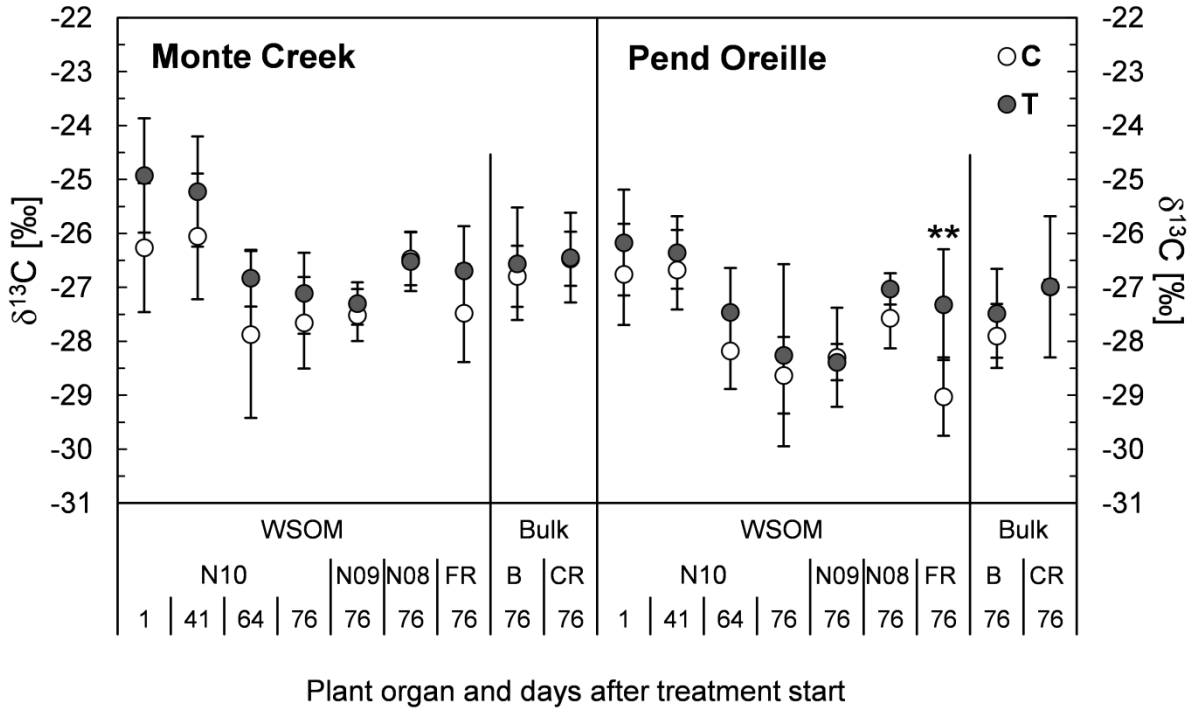

Figure 16 Effects of increased temperature and atmospheric drought on $\delta^{13}C$
$\delta^{13}C$ is shown for water-soluble organic matter (WSOM) in current year's needles (N10), older needles (N09, N08) and fine roots (FR), and in bulk material of bark and coarse roots (B, CR) from Douglas-fir seedlings of the provenances Pend Oreille and Monte Creek in control C (20°C, 0.35 kPa, white circles) and temperature/atmospheric drought treatment T (30°C, 1.91 kPa, grey circles). Asterisks indicate significant differences between treatments C and T (Tukey test, *** for p=0.001, ** p=0.01, * p=0.05). Data shown are mean values ± SD (N=3 (64 DAT) – 10 (76DAT)). Numbers 1, 41, 64, 76 in the x-axis caption indicate the time point of harvest after the onset of the experiment

Respiration rate and apparent respiratory fractionation

We determined canopy dark respiration rate and the difference between $\delta^{13}C$ of WSOM and CO_2 (apparent respiratory fractionation; $a\Delta_R$) during a diel course. There was no indication of a significant increase in $a\Delta_R$ during the light period (data not shown), revealing the absence of light enhanced dark respiration (Barbour *et al.*, 2007; Gessler *et al.*, 2009c). There was also no significant difference between dark respiration in the light and the dark phase, and therefore we calculated a daily average of the values (Figure 17).

Respiration rates did not differ significantly between treatment and control for both provenances and they did not show provenance-specific differences. In contrast, in both provenances $a\Delta_R$ decreased significantly with higher temperature and atmospheric drought by 2.2‰ for PO and 3.0‰ for MC. Although there was not a statistically significant difference, we observed a trend towards a lower $a\Delta_R$ in MC compared to PO independent of the treatment.

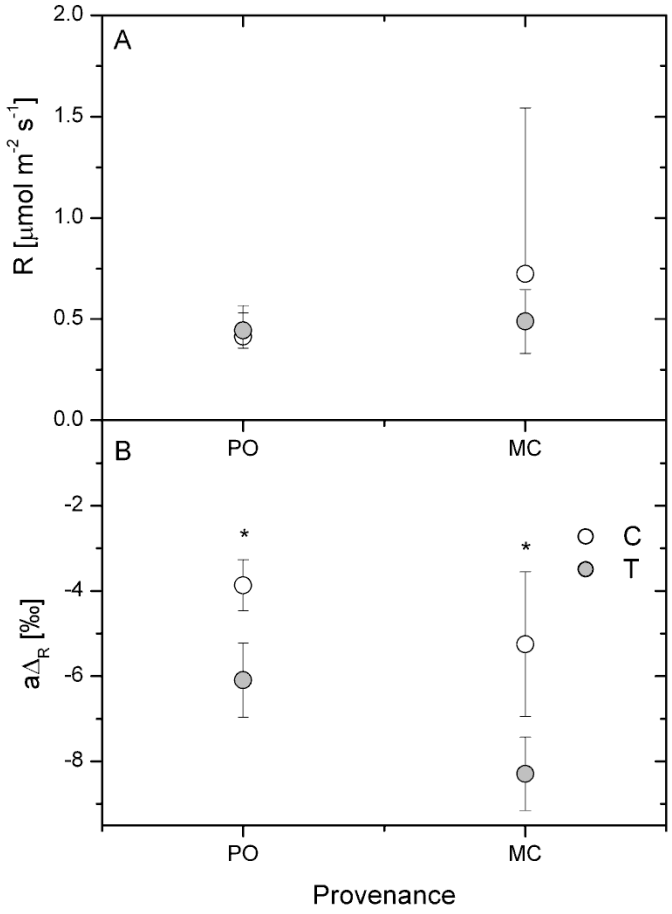

Figure 17 Canopy dark respiration rate (A) and apparent respiratory isotope fractionation ($a\Delta_R$) (B) in Douglas-fir seedlings of the provenances Pend Oreille (PO) and Monte Creek (MC)
The fractionation is based on the difference of $\delta^{13}C$ measured in water-soluble organic matter of needles and $\delta^{13}C$ in canopy respired CO_2 as average during a diel course. Data shown are mean values ± SE (N=3). Significance levels are given for differences between treatments as revealed by Student's t-test (* $p<0.05$). The controls (20°C, 0.35kPa) are indicated by white circles and the temperature/atmospheric drought treatment T (30°C, 1.91kPa) by grey circles

Metabolic profile

We analysed changes in the metabolite profiles due to the treatment in current-year (N10) and previous-year needles (N09) for the two provenances separately, and averaged over both provenances (Figure 18). In both provenances and both needle age classes, there was a general trend for an increase in monosaccharide concentrations in the high temperature treatment. The difference was significant for glucose in N10 needles of PO and for fructose, glucose and rhamnose in N10 needles averaged over both provenances. While sucrose concentrations did not change, raffinose decreased in both needle age classes (average of both provenances). Cellobiose increased in elevated temperature exposed plants in N10 in both provenances (sig-

nificant in PO) and as a consequence the average for the two provenances increased significantly. Within the group of polyols, myo-inositol decreased for the average of the two provenances in both age classes, but when analysed separately for each provenance, the decrease was only significant for MC. As a provenance average, D-pinitol and ononitol concentrations increased in both needle age classes and N10 needles, respectively, as a response to the treatment. These increases were most pronounced and significant in the provenance PO.

Organic acids involved in the TCA cycle were decreased upon the temperature and atmospheric drought treatment for the average of the two provenances: citrate and malate decreased in N10 and fumarate in N09 needles. This decrease was more prominent in the provenance PO and there was no significant difference between control and treatment in MC.

There was a trend for an increase in precursors and metabolites of secondary metabolite pathways as a consequence of the temperature treatment. This increase was significant for shikimate (N09, average of provenances; $p<0.1$), cinnamic acid (N10, PO; $p<0.05$), and quinic acid (N10, PO; $p<0.01$, and average of provenances; $p<0.001$).

For sugar acids, we observed an increase in saccharic acid under increased temperature and atmospheric drought (N10, MC), but a decrease in glyceric acid in both provenances (N10, MC, PO and average; N09, PO and average).

Total amino acid concentration decreased significantly in current-year needles (N10) in PO and as average of both provenances. The clearest decrease was observed for proline in N10 of both provenances whereas alanine increased in N09.

Flavonoids and especially catechin decreased significantly ($p<0.01$) and sitosterol concentrations increased in N09 needles ($p<0.01$) for the average of the two provenances.

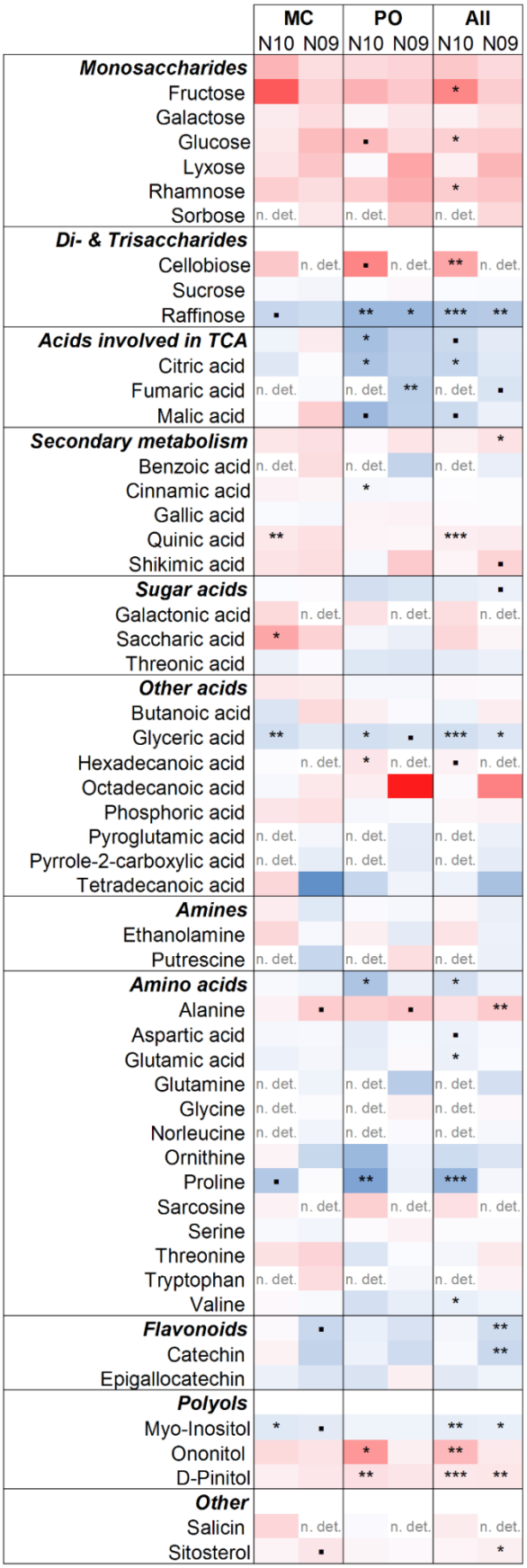

Figure 18 Effects of increased temperature and atmospheric drought on the metabolite profile of two Douglas-fir provenances (Monte Creek and Pend Oreille) and over all provenances (All) in current year's (N10) and last year's needles (N09)
Treatment effects are shown as the log10 ratios of temperature/atmospheric drought treatment (T) *vs.* controls (C) obtained from the mean values of the relatively quantified metabolite peaks of the two treatments (N=4-6 for for each treatment N10 and N=3-5 for N09). Significance levels are given for group differences (T vs. C) and were obtained by a post-hoc Tukey test (*** for p<0.001, ** p<0.01, * p<0.05, . p<0.1). n. det.: the compound was not detected in at least 50% of the replicates of each provenance and needle age class

4.5 Discussion

Plant biomass and $\delta^{13}C$ were only slightly affected by high temperature and atmospheric drought

In our study, the high temperature and atmospheric drought treatment (Table S 5) did not affect total biomass in the two Douglas-fir provenances examined (Figure S 6). We designed the experiment so that the new flush of needles was already fully developed by treatment initiation. Cambial activity is assumed to continue after leaf expansion, so that stem and root growth might be affected by the high temperature and atmospheric drought treatment. As expected, current-year needles biomass (N10) was not affected; the same was true for the previous-year needles (N09). The reduction in N08 needle biomass might be explained by needle loss during the experimental period and/or by withdrawal of non-structural compounds from senescing needles as accelerated senescence by heat stress has been observed previously (Thomas and Stoddart,1980). All other tissues, in which growth during the experimental period should be assumed (e.g. roots and stem tissues), were, however, not affected by the treatment and only slight changes in C allocation between current-year needles and fine roots, and between fine roots and coarse roots were observed (Figure S 6B).

At the same time, plant $\delta^{13}C$, as a marker for intrinsic water-use efficiency and the relationship between A_n and g_s, was largely unaffected (Figure 16). Even though our treatment clearly exceeded the optimum temperature for photosynthesis for Douglas-fir (≤ 20°C; Lewis et al. 2001) and we additionally induced atmospheric drought, neither the time-integrated relationship between A_n and g_s nor root and shoot biomass production were negatively affected during the treatment period. However, long-term effects of heat waves on growth remain to be investigated. Based on our observations over a 92-days treatment period, we reject our hypothesis (2) as our results point to the fact that the two provenances from the *menziesii-glauca* transition zone in southern interior British Columbia and the *glauca* zone demonstrate the physiological plasticity that allows the seedlings to continue assimilation and growth during extended periods of high temperatures in the late growing season.

The more negative apparent respiratory fractionation indicates switches in the substrate commitment from the TCA cycle to other metabolic pathways

We did not observe increased dark respiration rates under high temperature and atmospheric drought, which supports results from other studies, such as the work by Tjoelker et al. (1999), who found a strong thermal acclimation of respiration in conifers but not in broad-leaved species. In the control treatment, the isotopic enrichment of respired CO_2 above WSOM (as the putative substrate) was 3.9‰ and 5.2‰ in the Douglas-fir provenances PO and MC, respectively, and thus lies in the range observed in many other species (see review by Werner and Gessler 2011 and citations therein).

A decrease in the contribution of CO_2 decarboxylated in the TCA cycle relative to CO_2 released by PDH to total CO_2 production will cause an increase in $\delta^{13}C$ of respired CO_2 (Tcherkez *et al.*, 2003) and thus a decrease in $a\Delta_R$. Our results (Figure 17) thus point to the fact that from a given glucose moiety entering the glycolysis/TCA cycle relatively less CO_2 is evolved from the two TCA enzymes isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase in the high temperature treatment compared to the control. Thus, there were shifts in metabolic pathways in both provenances tested toward the production of secondary metabolites (and acetogenic lipids) derived from acetyl-CoA at the expense of a more complete oxidation of the initial glucose molecule in the TCA cycle.

High temperature induces a switch in stress protecting compounds from proline to polyols

Priault et al. (2009) hypothesised that differences in the apparent respiratory fractionation between different plant functional groups can be attributed to the relative quantity of carbon committed to the TCA cycle vs. to secondary metabolites and acetogenic fatty acids. These authors showed that Mediterranean species producing larger amounts of secondary metabolites showed more negative apparent respiratory fractionation as compared to species investing less in these compounds. In fact, our metabolite profiling shows that the concentrations of secondary compounds and partially unsaturated fatty acids (hexadecanoic (palmitic), octadecanoic (stearic) acid) tended to increase under high temperature and VPD at the expense of TCA intermediates. The relative changes in metabolite abundance of control and stressed plants together with the assessment of apparent respiratory fractionation provide general evi-

dence for altered metabolic priorities in both provenances under high temperature and atmospheric drought.

In general, the most abundant metabolites in all analysed tissues were sucrose, D-pinitol and quinic acid which is consistent with previous assessments of *Picea abies* (Riikonen *et al.*, 2012), *Pinus nigra* and *Abies alba* (Duquesnoy *et al.*, 2008). Although the sucrose level was not affected by the treatment, both pinitol and quinic acid clearly increased. Polyols such as pinitol and ononitol are involved in stress responses, such as drought and heat stress, and have also been associated with osmotic adjustment (Paul & Cockburn, 1989; Griffin *et al.*, 2004), scavenging of reactive oxygen species (ROS; Shen et al. 1997; Nishizawa et al. 2008), and osmoprotection (Hoekstra *et al.*, 2001; Taji *et al.*, 2002). Smirnoff and Stewart (1985) showed that among proline, betaine, sorbitol and mannitol, the polyols were most effective at increasing the stability of enzymes at high temperatures.

We infer that the observed increase in the two polyols is a direct acclimation response of the two Douglas-fir provenances examined to the potentially adverse effects of drought and high temperatures. In line with the increase in pinitol and ononitol is the decrease in myo-inositol and raffinose. Myo-inositol is a precursor for raffinose, ononitol and pinitol. The increased priority of polyol production most likely results in a decreased concentration of the precursor and thus potentially to restricted precursor availability for raffinose synthesis.

The increase in polyol synthesis is also related to the drop in proline concentrations under high temperature as observed in our study. Generally, proline accumulation is associated to drought and salinity stress (see e.g. Stewart *et al.*, 1980; Taylor, 1996), and proline functions as osmolyte, antioxidant, energy sinks and signalling molecule (Bouche & Fromm, 2004; Szabados & Savouré, 2010). Under these premises, we might expect proline to increase under high temperature and VPD. It has, however, also been observed that proline can be toxic to cells (Hellmann *et al.*, 2000; Deuschle *et al.*, 2001; Mani *et al.*, 2002; Nanjo *et al.*, 2003), and increased proline toxicity under heat was reported by Rizhsky *et al.* (2004). Furthermore, proline showed to be less efficient in osmoprotection at high temperatures (Smirnoff & Stewart, 1985), and in ROS scavenging (Buxton *et al.*, 1988) compared to polyols. We therefore conclude that there is a preference for the trees to produce pinitol and ononitol as ROS-scavengers and osmoprotectants at the expense of proline synthesis under high temperature. In

addition, the increase in alanine in N09 needles, which also may serve as osmolyte (Bouche & Fromm, 2004), might be seen in a similar light.

The concentration of monosaccharides in all analysed needle age classes (N10 and N09) increased under high temperature. Accumulation of monosaccharides was also observed in *Pinus* and *Larix* under drought (Sudachkova *et al.*, 2002) and in *Arabidopsis* under the combination of drought and elevated temperature (Rizhsky *et al.*, 2004) and after heat shock (Kaplan *et al.*, 2004). Similar to our results, Riikonen *et al.* (2012) found an increase of monosaccharides at the expense of trisaccharides in Norway spruce seedlings exposed to slightly elevated temperature. They concluded that the increase of monosaccharides results from the remobilization of stored resources to support growth at elevated temperature. However, monosaccharide accumulation might also reflect their role in a heat stress-specific replacement for proline as an osmoprotectant as previously suggested by Rizhsky *et al.* (2004) for *Arabidopsis*.

In our study, a large portion of identified metabolites in all treatments and provenances are related to the shikimate and phenylpropanoid pathways and the treatment effect across both provenances showed a significant increase in the total amount of acids involved in the shikimic acid pathway. We observed a general trend for increases in shikimic, quinic and galactonic acid in the high temperature treatment in both provenances. Elevated levels of quinic and shikimic acid were also reported in *Arabidopsis* in response to heat (Kaplan *et al.*, 2004). In contrast to our results, a decrease in phenolics at elevated temperature was detected in Douglas-fir (Riikonen *et al.*, 2012) and in other tree species (Kuokkanen *et al.*, 2001; Paajanen *et al.*, 2011). Quinic acid, related to phenylpropanoid synthesis, plant defence (Macheix *et al.*, 1990; Bennett & Wallsgrove, 1994), drought stress, and antioxidant activity (Rivas-Ubach *et al.*, 2012; Yang *et al.*, 2013), was one of the three most abundant metabolites in our study and increased significantly in current-year needles of the provenance MC grown under high temperature. This highlights an additional facet of the heat induced metabolic responses of Douglas-fir.

In conclusion, our results strongly support our hypothesis (1), indicating that high temperature and atmospheric drought alter the preference for specific metabolic pathways and the metabolite profiles. An array of metabolites protecting against high temperature, osmotic stress, and reactive oxygen species are accumulated as well as precursors of the secondary metabolism. Such accumulation seems to occur at the expense of proline. We might interpret the metabolic

acclimation as successful means to maintain physiological activity and functioning of Douglas-fir seedlings of the two examined provenances during heat waves. Concerning hypothesis (3), we can clearly state that there are no differences in biomass accumulation of seedlings from both provenances (Monte Creek and Pend Oreille). Even though the effects of increased temperature combined with atmospheric drought on the metabolism might have been slightly different between the two provenances, a general parallel trend could be observed. Apparently, the metabolic mechanisms involved in mediating resistance to elevated temperature and atmospheric drought are similar in the two provenances tested here, and we can thus reject hypothesis (3). The two provenances originated from rather dry interior areas of origin (Table 13) and we might assume that coastal provenances adapted to a more humid climate respond differently and are more sensitive to heat and drought. However, Jansen et al. (2013) observed no relation between drought sensitivity and the environmental conditions at the site of origin for old-growth coastal Douglas-fir. They concluded that long-term climatic conditions at the origin averaged over the year or the growing season are poor indicators for the occurrence of stress and that the frequency of heat and drought events in a region might be better predictors for adaptation. Based on the results of the two provenances presented here, we might extend this hypothesis to seedlings. In conclusion, we can state that the Douglas-fir seedlings from the *glauca* zone and *menziesii-glauca* transition assessed here are well adapted to extended periods of high air temperature and atmospheric drought and thus might represent a suitable resource for forestry in Central Europe under future climatic conditions and extreme events.

4.6 Acknowledgements

We thank Nursery Services Interior, Vernon, and Webster Forest Nursery, Olympia, for intensive consulting and seedling supply, as well as Robert Hommel and Christine Ewald for assistance during the experimental work. We acknowledge Susanne Remus for her assistance with the isotopic analysis.

4.7 Supplementary Material

Table S 5 Growing conditions during the acclimatization and treatment phase in the walk-in climate chambers with the control (C) and the treatment (T) conditions

	Acclimatization phase [d before treatment start]				Treatment phase [d after treatment start]
	-78	-72	-68	-3	0-92
Photosynthetic photon flux density [µmol m^{-2} s^{-1}]	250 ±50	250 ±50	250 ±50	400 ±50	600 ±50
Photoperiod [h day^{-1}]	12	14	16	16	16
Air temperature [°C day/night]	10/10	15/13	20/15	20/15 (C)	20/15 (C)
				25/20 (T)	30/25 (T)
Relative air humidity [% day]	85 ±5	85 ±5	85 ±5	85 ±5 (C)	85 ±5 (C)
				65 ±5 (T)	55 ±5 (T)
Air vapour pressure deficit [kPa]					0.35 ±0.1 (C)
					1.91 ±0.2 (T)

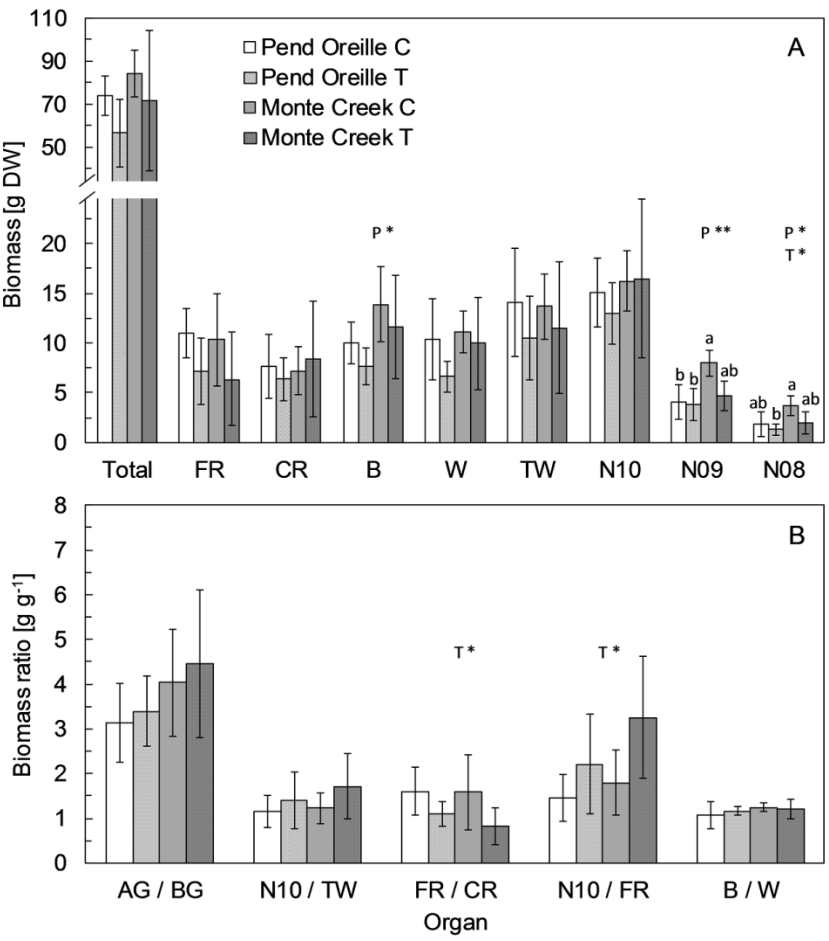

Figure S 6 Effects of increased temperature on Douglas-fir provenances total and organ-specific biomass (A) and on biomass ratios between organs (B)
(A) shows absolute dry weight data of seedlings of the provenances Pend Oreille and Monte Creek grown under control conditions (C; 20°C, 85% rH) and elevated temperature and increased VPD (T; 30°C, 55% rH). Data shown are mean values ± SD (N=4 to 5). Small letters indicate homogeneous groups (Tukey posthoc test). Large letters indicate significant effects of provenance (P) and treatment (T) (ANOVA). FR, fine roots, CR, coarse roots, B, bark, W, wood, TW, twig, N10, current year needles (2010), N09 and N08, previous year needles (2009, 2008, respectively)

5 Synthesis: Sensitivity and resistance towards drought and heat stress in different Douglas-fir provenances

5.1 Application and Interpretation of Stress Parameters

Tree stress responses occur at different levels and in an interactive way. We measured leaf gas exchange and associated parameters such as leaf isotopic composition (chapter 1) that respond to water and light availability and have implications for carbon assimilation and thus wood growth (chapter 3). At the same time, gas exchange is interlinked with protective plant mechanisms such as osmotic adjustment (chapter 1), changes in metabolic pathways (chapter 3 and Du *et al.,* 2014, see appendix) and non-photochemical quenching (Junker *et al.*, 2017, see appendix). Hence, if we look at single parameters in order to test the stress response, we lose information on interactions in the plants response, and might even misinterpret response patterns in terms of stress sensitivity or resistance (for a condensed overview on these mechanisms see Figure 1).

Moreover, time scales are important to distinguish between short term and long term responses in long-living organisms such as trees. Consequently, we applied parameters that i) report responses at the exact time point of measurement (gas exchange), ii) integrate responses over few days up to a growing season (leaf isotopic composition in different fractions of the leaf), and iii) report responses retrospectively in years or decades (isotopic composition and width of tree rings).

5.1.1 Leaf gas exchange and isotopic composition in different plant tissues

Plants respond to environmental stress with various levels of stress responses, and, having a sessile life style, have developed strategies of stress tolerance and stress avoidance, both leading to stress resistance, a term first defined by Levitt (1980) in agreement with the knowledge of physiology at that time. Traits leading to higher stress tolerance include for instance the ability to function under (environmental) drought or, more specifically, while dehydrated (physiological drought). One example for tolerance towards cell dehydration is the synthesis

of organic solutes that protect cellular proteins. In contrast, stress avoidance mechanisms maintain for instance the water balance in the plant. Definitions for stress resistance strategies are, however, still not clear and used without consensus in literature (Blum, 2016). For instance, leaf osmotic adjustment is on the one hand associated to drought tolerance in many studies (Touchette *et al.*, 2007; Kooyers, 2015), enabling the plants to maintain their gas exchange under environmental drought (and thus seemingly tolerating drought). On the other hand, it avoids physiological drought by maintaining leaf turgor under drought stress (Hasegawa *et al.*, 2000; Chen *et al.*, 2007; Cuin *et al.*, 2010) and might be considered a drought avoidance trait (Blum, 2016). Stomatal closure is often associated to avoidance of physiological drought (Franks, 2011) whereas the investment into fine root production avoids environmental drought to some extent, improving the exploitation of soil water resources in dry periods (Meier & Leuschner, 2008). Further confusion is created by different research aims: A strong stomatal response to drought is considered a trait of drought avoidance by plant physiologists as it maintains leaf water potential and xylem conductivity, but the trade-off, reduced carbon assimilation, is mostly associated with growth declines that are generally considered a trait of drought sensitivity from the wood growth perspective. There is a need to distinguish between different research aims, and between external stress and internal strain, and the respective point of view taken by a researcher determines the questions raised and interpretations found by a study. In the following, I will use the term 'stress resistance', and most of all focus on describing specific responses to internal strains without associating plant traits to either stress 'tolerance' or 'avoidance'.

Responses to water deficit (in soil and plant) typically involve, as an early response, the regulation of stomatal conductance to water vapour (g_s) reducing water loss through transpiration but at the same time reducing the leaf intercellular CO_2 concentration (c_i) and thus CO_2 supply for photosynthesis (Chaves, 1991; Cornic & Massacci, 1996). Changes in g_s and assimilation rate (A_n) can be assessed by instantaneous gas exchange measurements on the (living) leaf, and can be used to derive the intrinsic water-use efficiency (IWUE) as the ratio of A_n to g_s. The effects of changes in g_s and A_n on c_i are imprinted onto $\delta^{13}C$ of recent assimilates (Farquhar *et al.*, 1982; Brugnoli *et al.*, 1988; Cernusak *et al.*, 2005), rendering $\delta^{13}C$ an integrative parameter for IWUE (Farquhar *et al.*, 1989).

The water-soluble organic matter (WSOM) of leaves has proved to be an excellent proxy for sugars and thus recent assimilates (Gessler *et al.*, 2009c) and $\delta^{13}C$ in leaf WSOM integrates IWUE over a period of hours to days (Keitel et al., 2003; Brandes et al., 2006). The isotope signal of recent assimilates is transferred to the total organic matter (TOM) of leaves over longer time periods. Leaf TOM integrates over several weeks or months up to the whole lifespan of the leaf. Correlation analyses showed that leaf $\delta^{13}C_{TOM}$ (sampled in September) integrated physiological responses of Douglas-fir trees towards weather conditions during the summer months rather than during spring (chapter 2; Figure 7) pointing to reserves strongly affecting the total organic carbon (and its isotopic signature) in Douglas fir needles.

Different methods revealed concurring results for IWUE in Douglas-fir provenances growing at a humid field site (chapter 2): We observed a correlation between $\delta^{13}C_{WSOM}$ (as a proxy for recent assimilates) and instantaneous leaf gas exchange data at the humid field site Schluchsee (Table 7). Furthermore, we derived information on IWUE integrating over the whole growing season from gas exchange measurements and from $\delta^{13}C$ in leaf WSOM and TOM: We used $\delta^{13}C_{TOM}$ in current-year needles sampled at the end of the growing season ($IWUE_{13C\ TOM}$, Figure 5), previous-year needle $\delta^{13}C_{WSOM}$ averaged over sampling time points from May to September ($IWUE_{13C\ WSOM\ AVG}$), the ratio of A_n and g_s assessed by gas exchange measurements and averaged over all time points ($IWUE_{GE\ AVG}$), and the regression between all measured A_n and g_s, considering the slope of the regression line as an integrating IWUE over the whole measurement period ($IWUE_{GE\ slope}$). These results show that under humid site conditions, the use of isotopic data reveals physiological information in Douglas-fir trees according to isotope theory (Farquhar et al., 1982).

For the dry field site Wiesloch, however, the information derived from the different methods was not closely related to each other (Table 7). Here, we observed a small operational range of stomata with g_s and A_n at comparably low levels (Junker *et al.*, 2017), going in line with a low sensitivity of $\delta^{13}C_{WSOM}$ to environmental conditions (chapter 2, Figure 8). Therefore, the observed lack of correlation between $IWUE_{13C}$ and $IWUE_{GE}$ at Wiesloch might be linked to the limited range of both parameters at this dry field site. At the same time our results indicate osmotic adjustment (OA) at the dry field site (see chapter 2, Figure 9). OA might also play a role but should, in theory, affect $IWUE_{13C}$ and $IWUE_{GE}$ equally via the effect on leaf water status (probably explaining the observed low sensitivity of $\delta^{13}C_{WSOM}$ to environmental condi-

tions, see chapter 5.1.3). A highly variable contribution of assimilates produced during lower light intensities at the site Wiesloch would, however, also explain the differences between $IWUE_{13C}$ and $IWUE_{GE}$ (see chapter 2). If that is the case, the integrating isotope-based measure represents the "effective" IWUE better than point measurements at a given light (and temperature) level.

Significant portions of the isotope signal of recent assimilates are furthermore transferred to the tree-ring structural organic matter (Gessler *et al.*, 2009a) which is an ongoing process over the growing season. Nevertheless, tree ring growth during early parts of the growing season can depend on carbon reserves especially in deciduous but also in evergreen coniferous species (Helle *et al.*, 2004). Therefore, we excluded the early wood portion of tree rings from isotope analyses (chapter 3). Our study on three field sites in Southwest Germany showed that late wood $\delta^{13}C$ increased in all provenances and at all field sites during the severe and prolonged drought in the year 2003. Nevertheless, Douglas-fir provenances varied in their drought response at the dry valley site as assessed by the level of radial growth decline and the increase in late wood $\delta^{13}C$ (Figure 15). To disentangle stomatal and photosynthetic effects on $\delta^{13}C$, a dual isotope approach based on carbon and oxygen isotope composition in plant organic matter is often applied (Scheidegger *et al.*, 2000; Barbour *et al.*, 2002; Ripullone *et al.*, 2009; Gessler *et al.*, 2009a; Barnard *et al.*, 2012) as $\delta^{18}O$ is in contrast to $\delta^{13}C$ only related to g_s but not to photosynthesis. Combined measurements of $\delta^{13}C$ and $\delta^{18}O$ thus allow a separation between stomatal and photosynthetic effects on $\delta^{13}C$ (Scheidegger *et al.*, 2000; Barbour *et al.*, 2002; Ripullone *et al.*, 2009; Gessler *et al.*, 2009a; Barnard *et al.*, 2012) with some restrictions and precautions to be taken into account as described by Roden and Siegwolf (2012). We applied the dual isotope approach in tree ring late wood (chapter 3) and were able to link the radial growth decline and the increase in late wood $\delta^{13}C$ and $\delta^{18}O$ from 2002 (year with average temperature and precipitation) to 2003 (dry and hot year) (Figure 15) to a decrease in g_s in four coastal provenances at the field site in the valley (Figure 14). This field site is within the altitudinal gradient the warmest and driest and thus it is highly reasonable that the exceptional hot drought in 2003 had the strongest effect there. Comparable i.e. strongest effects of the 2003 heatwave at valley sites in altitudinal gradients have been also observed in other studies (e.g. Dobbertin 2005).

Among the provenances tested, Conrad Creek displayed the largest radial growth decline and the strongest increase in tree ring $\delta^{13}C$ and consequently IWUE in response to the severe drought. This increase in IWUE is attributed to stomatal closure as assessed by the dual isotope approach. A similar strong response became apparent with our instantaneous gas exchange measurements on leaves at the field site Schluchsee: From the humid conditions in July 2010 to the very dry May 2011, the strongest decline in g_s was observed in this provenance (Figure S1 in Junker *et al.*, 2017), indicating (1) concurrent results in instantaneous and retrospective methods and (2) pointing to the fact that the coastal provenance Conrad Creek shows the most sensitive stomatal response to drought.

5.1.2 Stomatal and growth responses to drought and elevated temperature

Decreases in g_s reduce transpiration and thus water loss but at the same time limit CO_2 diffusion to the chloroplasts and can thus lead to a decline in carbon assimilation and growth. After extreme events the growth decline can be followed by an increasing mortality as observed during the heat and drought in 2003 in Europe (Ciais et al., 2005; Bréda et al., 2006; Allen et al., 2010). A strong (early) stomatal response to drought (isohydric regulation of G_s) maintains leaf water potential and xylem conductivity under reduced carbon assimilation (Hubbard *et al.*, 2001; Mcculloh *et al.*, 2014). Anisohydric plants in contrast allow a longer maintenance of high stomatal conductance and photosynthesis under drought, risking water loss and xylem cavitation (Sade *et al.*, 2012).

The hydraulic failure-carbon starvation framework (McDowell *et al.*, 2008) specifies why trees die from drought: Plants with isohydric regulation of water status avoid drought-induced hydraulic failure, resulting in carbon starvation and eventually in reduced resistance to biotic agents. Hydraulic failure is associated to plants operating with narrower hydraulic safety margins during drought, such as seedlings, some anisohydric plants and trees near their maximum height. Regarding intra-specific variability of drought resistance in isohydric species, rather anisohydric genotypes are often discussed as drought sensitive, showing increased mortality under severe drought due to a high water loss (Moshelion *et al.*, 2014).

However, the comparably drought-tolerant species Douglas-fir shows is often assumed to show rather anisohydric regulation of stomatal conductance in combination with cavitation resistant wood properties (Anekonda et al., 2002; Warren et al., 2003) and leaf anatomical and

biochemical traits associated with leaf longevity and drought resistance (Warren & Adams, 2004). Also hydraulic redistribution, the passive water movement via roots from moist to drier portions of the soil, has been shown to be an important mechanism for maintaining shallow root function during drought and preventing total stomatal closure in young and especially in old-growth Douglas-fir (Domec *et al.*, 2004). This species can sustain a high long term growth potential on warm and dry sites as shown at the field site Wiesloch in Southwest Germany (see chapter 2 and (Neophytou *et al.*, 2016; Junker *et al.*, 2017).

Elevated temperature may stimulate assimilation and growth below the thermal optimum, but may lead to reduced performance above species- or provenance-specific thresholds (Rehfeldt *et al.*, 2002; Ryan, 2010). Evergreen species have been reported to show either a weak growth increase compared to deciduous tree species (Way and Oren 2010) or no growth response to moderate temperature increases (Carter 1996). Depending on field site elevation, the extreme and prolonged heat and drought wave in 2003 in Europe showed diverging effects on radial growth in Douglas-fir, ranging from no response at moist sites to severe decreases at dry sites (see our study in chapter 3 and Lévesque *et al.* (Lévesque *et al.*, 2014). We linked the growth decline 2003 at a valley site in Southwest Germany to a decrease in g_s as assessed by the relations between $\delta^{18}O$ and $\delta^{13}C$ in tree rings of four coastal provenances (chapter 3). We therefore conclude that the limited water supply (possibly mediated by higher temperature and higher VPD) was the main factor for the growth decline in 2003.

Highly variable water availability during the years 2010 and 2011 at a high elevation site in Southwest Germany (Schluchsee; 1050 m asl) led to a large range in g_s and a strong link between water availability, stomatal conductance and leaf carbon isotope composition in Douglas-fir (chapter 2). Annual precipitation in 2010 and 2011 (Table 5) dropped clearly below the long term average conditions (Table 4) by -592 and -874 mm, respectively. Gas exchange measurements showed strongly decreased g_s under the severely reduced water availability in spring 2011. The trees recovered and showed very high g_s again in summer 2011 (Junker *et al.*, 2017). Correlations with weather conditions revealed that leaf carbon isotope composition was strongly coupled to water availability (Figure 7 & 8). These results suggest an isohydric regulation of g_s under strongly reduced water availability at a generally humid field site. (McDowell et al., 2008) suggested that wet periods stimulate shifts in hydraulic architecture predisposing plants to water stress. In our study, 50-years old Douglas-fir trees growing under

generally wet conditions were more responsive to drought in terms of their stomatal regulation than trees growing at the generally dry field site Wiesloch. Here, gas exchange measurements (Junker *et al.*, 2017) showed a small range in g_s during the growing seasons 2010 and 2011, when annual precipitation sum remained below mean annual precipitation by -250 and -381 mm, respectively (Table 5 & 6). At Wiesloch a rather anisohydric regulation of g_s becomes apparent under constantly dry conditions. We found one potential underlying mechanism, osmotic adjustment, supporting this anisohydric behaviour as discussed in chapter 2 and 5.1.3.

However, our results from the extreme drought in 2003 (Mooswald, chapter 3) and under strongly reduced water availability in 2011 (Schluchsee, chapter 2) show tipping points in the trees' response patterns: When water availability differs extremely from average conditions at a given site, Douglas fir strongly reduces stomatal conductance, diverging from the generally observed anisohydric regulation of water status in this species.

5.1.3 Osmotic adjustment

In plant response to heat and drought stress, osmotic adjustment (OA) can increase stress resistance by regulating plant cell turgor via synthesis of organic osmolytes, and by controlling ion fluxes through cellular membranes. Organic osmolytes such as glycine betaine, proline and polyols are accumulated under drought or salinity conditions (Bohnert et al., 1995; Hasegawa et al., 2000). The polyol D-pinitol is known to be present in large amounts in conifers (e.g. Riikonen et al. 2012) and might play an important role in osmotic adjustment (Paul & Cockburn, 1989; Griffin *et al.*, 2004).

In an experiment under controlled conditions (chapter 4), Douglas-fir saplings were exposed to a combined treatment of heat and atmospheric drought. The saplings accumulated an array of metabolites protecting against high temperature, osmotic stress, and reactive oxygen species (Figure 18). The polyols ononitol and D-pinitol, the amino acid alanine, and several monosaccharides increased under heat and atmospheric drought, and may serve as organic osmolytes (Paul & Cockburn, 1989; Bouche & Fromm, 2004; Griffin *et al.*, 2004; Rizhsky *et al.*, 2004). Total biomass (Figure S 6 in chapter 4) and plant carbon isotope composition, as a marker for intrinsic water-use efficiency (Figure 16), were largely unaffected. Thus, the metabolic acclimation including osmotic adjustment might serve as successful means to maintain

physiological activity and functioning of Douglas-fir seedlings of the two examined provenances Pend Oreille and Monte Creek during heat waves.

Interestingly, the concentration of total amino acids decreased under heat and atmospheric drought (chapter 4). The clearest decrease was observed for proline although it has been associated to various environmental stresses (Stewart et al. 1980; Taylor 1996). The decrease in proline levels in our study might be explained by its potential cell toxicity (Hellmann et al. 2000; Mani et al. 2002), especially under heat (Rizhsky et al. 2004). Our results thus point to a switch from proline to polyols as osmolytes in Douglas-fir seedlings under a simulated heat wave.

However, the biosynthesis of organic osmolytes is an expensive process. The accumulation of inorganic ions such as Na^+, Cl^- and K^+ is an alternative, contributing greatly to osmotic adjustment (Chen et al., 2007; Cuin et al., 2010; Shabala & Shabala, 2011). OA by accumulation of ions might explain our results at the dry field site Wiesloch in 2010 and 2011 (105 m asl, chapter 2): The carbon isotope composition in leaf water-soluble organic matter ($\delta^{13}C_{WSOM}$), a proxy for sugars and thus recent assimilates (Gessler *et al.*, 2009c), was not related to water availability at this site (Figure 8), as shown by correlations with precipitation, total available soil water and relative humidity of the air during one to 20 days prior to leaf sampling. Furthermore, stomatal control of $\delta^{13}C$ was low as assessed by the correlation analysis between $IWUE_{13C}$ and g_s (chapter 2, Table 7). The isotopic data points to stomata operating over a limited range which was confirmed by gas exchange measurements (Junker *et al.*, 2017). At the same time, leaf mineral and water content both increased under drier conditions at Wiesloch, indicating osmotic adjustment (chapter 2, Figure 9). We conclude that the maintenance of plant cell turgor by OA might be part of the explanation for the low responsiveness of g_s and $\delta^{13}C$ to water availability at this field site. Furthermore, osmotic adjustment might be one of the mechanisms underlying an anisohydric regulation of g_s in Douglas-fir trees growing in a generally dry environment.

5.1.4 Photoprotective mechanisms

Stomatal closure reduces water loss from the leaf, but at the same time decreases the leaf internal CO_2 concentration. In C3 plants, the photosynthetic CO_2 fixation is impaired under these conditions, and with it the metabolic sink for electrons generated during the light reac-

tions. Thus, light energy cannot be quenched photochemically and might support the formation of cytotoxic reactive oxygen species (ROS; (Niyogi, 2000; Ensminger *et al.*, 2006). Non-photochemical quenching (NPQ), scavenging of ROS and production of volatiles are the main photoprotective mechanisms in plants to avoid damage from excessive light energy (Baroli & Niyogi, 2000; Munekage et al., 2002; Peñuelas & Munné-Bosch, 2005).

The accumulation of ROS scavenging compounds was observed in Douglas-fir saplings subjected to a simulated heat wave (Figure 18, chapter 4): The polyols pinitol and ononitol, associated with ROS scavenging (Shen *et al.*, 1997; Nishizawa *et al.*, 2008), and quinic acid, related to antioxidant activity (Rivas-Ubach *et al.*, 2012; Yang *et al.*, 2013), increased significantly under high temperature and atmospheric drought. Intrinsic water-use efficiency (IWUE, assessed by plant $\delta^{13}C$; Figure 16) increased significantly in one plant compartment in one provenance and increased by trend in all other compartments analysed. An increase in IWUE, as the ratio between A_n and g_s, is associated to increases in A_n and/or decreases in g_s. The latter would explain the observed high abundance of ROS scavenging and antioxidant compounds under high temperature and atmospheric drought. Finally, plant growth was only marginally affected by the treatment (Figure S 6), showing the potential of the studied Douglas-fir saplings to cope with extended periods of high air temperature and atmospheric drought.

Another photoprotective mechanism, NPQ, can be assessed by the xanthophyll cycle pigments (VAZ) pool size and its deepoxidation state (DEPS; (Adams & Demmig-Adams, 1994; Ensminger et al., 2004; Jahns et al., 2009). Junker et al. (2017) reported higher DEPS and monoterpene emissions at Wiesloch compared to Schluchsee during the growing seasons 2010 and 2011 (Figure 3 therein), indicating that instantaneous photoprotective mechanisms (Niyogi, 2000; Demmig-Adams & Adams, 2006) are more pronounced at the dry field site Wiesloch. The provenances also differed in their photoprotective mechanisms as described in chapter 5.2.

5.1.5 Responses to heat assessed by apparent respiratory carbon isotope fractionation and metabolite profiling

Changes in temperature may alter the carbon allocation to different metabolic pathways (Kaplan *et al.*, 2004). Shifts in the commitment of metabolic pathways can be detected by

changes in the apparent respiratory carbon isotope fractionation (Werner & Gessler, 2011), which shows the level of ^{13}C-enrichment of leaf respired CO_2 compared to the putative respiratory substrate. During respiration of glucose molecules, the pyruvate dehydrogenase (PDH) reaction releases ^{13}C-enriched CO_2 (Tcherkez *et al.*, 2003). The remaining part of the pyruvate molecule can either enter the tricarboxylic acid (TCA) cycle, during which relatively depleted CO_2 is released, or might be used for the synthesis of lipids and various secondary metabolites (Werner & Gessler, 2011). The more pyruvate is committed to metabolic pathways other than the TCA cycle, the higher the contribution of the PDH-released ^{13}C enriched CO_2 to the total respiratory CO_2 emission, and the more negative the apparent respiratory fractionation ($a\Delta_R$). Leaf respired CO_2 is generally ^{13}C-enriched compared to the putative respiratory substrate but $a\Delta_R$ is variable among species and with environmental conditions (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Werner & Gessler, 2011). In our study $a\Delta_R$ ranged between -4 and -5 ‰ in young Douglas-fir saplings (controls, chapter 4). Under elevated temperature, an increase in respiration and TCA activity could be expected, leading to a less negative $a\Delta_R$. We, however, observed the opposite effect: When exposed to a combined treatment of heat and atmospheric drought for 64 days, the saplings showed no change in dark respiration rates which could be linked to thermal acclimation of respiration that was also reported by Tjoelker *et al.* (1999) for conifers but not for broad-leaved species. Furthermore, we observed significantly decreased $a\Delta_R$ (by 2.2 and 3.0 ‰ in the Douglas-fir provenances Pend Oreille and Monte Creek, respectively), indicating a switch in the substrate commitment from the TCA cycle to other metabolic pathways such as secondary metabolism.

To obtain information about the accumulation of precursors of secondary metabolites or stress related compounds we analysed metabolite profiles. Increased levels of monosaccharides in Douglas-fir exposed to high temperature and atmospheric drought (chapter 4) in our study as well as in Norway spruce under elevated temperature (Riikonen *et al.*, 2012), and in *Arabidopsis* under combined drought and elevated temperature (Rizhsky *et al.*, 2004) might reflect their role in a heat stress specific replacement for proline as an osmoprotectant as previously suggested by Rizhsky et al. (2004) for *Arabidopsis*. Our study on Douglas-fir as well as the study on Norway spruce (Riikonen *et al.*, 2012) showed that monosaccharides increased at the expense of trisaccharides under elevated temperature. Riikonen *et al.* (2012) explained this with the remobilization of monosaccharides from stored resources to sustain growth un-

der elevated temperature. However, our metabolic analyses indicated that the significant decrease in the concentration of the trisaccharide raffinose resulted from significantly decreased myo-inositol levels, which is a precursor not only for raffinose but also for ononitol and pinitol synthesis. At the same time, the simulated heat wave increased polyol levels (chapter 4 and discussion above) that have been associated with osmoprotection (Hoekstra *et al.*, 2001; Taji *et al.*, 2002) and stabilization of enzymes at high temperatures (Smirnoff & Stewart, 1985). The increased priority of polyol production under heat most likely leads to a decreased concentration of the precursor and thus to restricted precursor availability for raffinose synthesis.

Secondary metabolites related to the shikimate pathway such as quinic and shikimic acid (Kaplan *et al.*, 2004) are linked to the synthesis of aromatic amino acids, phenylpropanoids, flavonoids, and other metabolites (Buchanan *et al.*, 2000), and are generally upregulated under heat (Kaplan *et al.*, 2004). Our experiment on Douglas-fir saplings confirmed this finding (Figure 18). The simulated heat wave significantly increased the total amount of acids involved in the shikimic acid pathway and significantly increased the levels of quinic acid, which is related to phenylpropanoid synthesis, plant defence (Macheix *et al.*, 1990; Bennett & Wallsgrove, 1994), and drought stress. Quinic acid was furthermore one of the most abundant metabolites in Douglas-fir (chapter 4), Norway spruce (Riikonen *et al.*, 2012), black pine and silver fir (Duquesnoy *et al.*, 2008).

In summary, the relative changes in metabolite abundance together with the assessment of apparent respiratory fractionation provide general evidence for altered metabolic priorities in Douglas-fir saplings under high temperature and atmospheric drought. Our results demonstrate the great value of both methods for assessing plant metabolic responses to changing environmental conditions. Furthermore, these shifts in metabolic pathways can be regarded one important suite of mechanisms to explain the resistance of Douglas-fir to global change.

5.2 Provenance-specific stress sensitivity and resistance

By applying bioclimate envelope models developed for North America to climate change scenarios for Western Europe, Isaac-Renton *et al.* (2014) predicted the best performing prove-

nances that have shown highest growth potential in field trials in the period 1961-1990. Coastal Washington provenances from both high precipitation coast areas and from relatively drier inland areas have been top performers across Western Europe. Our results from provenance trials in Southwest Germany support this finding when studying provenances under average conditions (chapter 2 & 3; Junker *et al.*, 2017, see appendix) but we cannot confirm this result regarding drought conditions. Isaac-Renton *et al.* (2014) also demonstrated that climate observations over the last three decades justify changes to current use of Douglas-fir provenances in plantation forestry in western and central Europe. In the experiments at field sites and under controlled conditions in climate chambers, we studied coastal, transition and interior provenances (Table 2) and their metabolic, physiological and morphological responses to average and extreme conditions to provide a more clear picture about the drought resistance and the underlying mechanisms of different Douglas-fir provenances.

5.2.1 Coastal Douglas-fir provenances

During the extreme drought and heat in 2003, coastal Douglas-fir provenances clearly varied in their drought response at the dry valley site in Southwest Germany as assessed by the radial growth decline and the increase in tree ring $\delta^{13}C$ (Figure 15). Long term growth performance of a provenance might not be related to the growth response to drought stress and thus long-term economic value might need to include risk estimates of tree performance under climate extremes. Indeed, we observed a trade-off between the response to extreme events and the growth potential under moderate environmental conditions in two provenances.

Table 14 Ranking among four coastal Douglas-fir provenances in their long term height growth studied at six field sites in Southwest Germany (data compiled from Jansen *et al.* (2013) and Neophytou *et al.* (2016)

Field site	Wiesloch (Dgl122) *(Neophytou et al., 2016)*	Mooswald (Dgl115) *(Jansen et al., 2013)*	Sindelfingen (Dgl191) *(Neophytou et al., 2016)*	Illenberg (Dgl114) *(Jansen et al., 2013)*	Schauinsland (Dgl116) *(Jansen et al., 2013)*	Schluchsee (Dgl123) *(Neophytou et al., 2016)*	Average rank ± SD
Elevation (m asl)	105	230	490	500	940	1050	
Cameron Lake	4	1	3	2	3	1	2.3±1.2
Conrad Creek	1	2	1	1	2	2	1.5±0.5
Timber	2	3	2	3	1	3	2.3±0.8
Santiam River	3	4	4	4	4	4	3.8±0.4

Superior long term height growth under average conditions at different elevation levels was observed in the coastal provenance Conrad Creek (Table 14), which originates from the region with the second highest mean annual and summer precipitation amongst the provenances tested here (2390 and 415 mm, respectively, Table 2).

The isotopic data reveals high intrinsic water-use efficiency (IWUE) in this provenance at the field sites Wiesloch and Schluchsee: The parameter for IWUE, carbon isotope composition in leaf WSOM ($\delta^{13}C_{WSOM}$), was significantly highest in Conrad Creek under average water supply in July 2010 at Schluchsee (corresponding to lowest carbon isotope discrimination; Figure 1 & S1 in Junker *et al.*, 2017; see appendix). Also long-term integrating measures indicated that over the two growing seasons assessed intrinsic water-use efficiency was highest in Conrad Creek among four provenances (by trend but not significant; Figure 5; $IWUE_{GE\ slope}$ and $IWUE_{13C\ WSOM\ AVG}$). Net carbon assimilation rate (A_n) was by trend highest in Conrad Creek among three coastal provenances at Schluchsee (not sign.; Figure 2 in Junker *et al.*, 2017). At the dry valley site Wiesloch, Conrad Creek showed significantly highest $\delta^{13}C_{WSOM}$ during average conditions (Post-hoc Tukey multiple comparisons between provenances overall time points in 2010 and 2011, Table S 2) as well as during dry and wet periods (July 2010 and 2011, respectively; Figure S1 in Junker *et al.*, 2017). Even though these results could not be confirmed by gas exchange measurements at Wiesloch (Figure 2 & S1 in Junker *et al.*, 2017) the data point to the high growth and physiological potential.

In summary, the isotopic data points to high IWUE of Conrad Creek at both field sites and under various environmental conditions. At Schluchsee this result is confirmed by gas exchange data on IWUE and can be linked to the highest carbon assimilation rates observed in this provenance, potentially explaining the high growth potential of Conrad Creek under average conditions (for an overview see Table 15).

However, during the extreme drought and heat in 2003, Conrad Creek displayed the largest radial growth decline from 2002 to 2003 and the strongest increase in tree ring $\delta^{13}C$ and IWUE at the valley site Mooswald (Figure 15), which was attributed to stomatal closure as assessed by a dual isotope approach (Figure 14). A similar drought response became apparent applying instantaneous gas exchange measurements on leaves: From the humid conditions in

July 2010 to the very dry May 2011 at the field site Schluchsee, the strongest decline in g_s was observed in Conrad Creek (Figure S1 in Junker *et al.*, 2017). A strong stomatal response to drought points to an isohydric regulation of g_s in this provenance (for an overview on drought responses see Table 16).

Junker *et al.* (2017) reported high VAZ pool sizes and a positive relation between DEPS and sunshine hours in Conrad Creek, indicating an instantaneous mechanism to dissipate excess light energy as heat and facilitating NPQ (Niyogi, 2000; Demmig-Adams & Adams, 2006). However, the pigment composition of the photosynthetic apparatus is adjusted on longer timescales and is linked to species-specific adaptation to environmental conditions (Ensminger et al., 2004; Croce & van Amerongen, 2014; Fréchette et al., 2015). An intermediate chlorophyll content and carotenoid-chlorophyll ratio under drought in Conrad Creek (Figure 6 in Junker *et al.* (Junker *et al.*, 2017)) thus point to medium levels of (long term adjusted) photoprotection (Duan et al., 2005; Baquedano & Castillo, 2006; Gallé et al., 2007).

Conrad Creek responds strongly to drought by stomatal closure but at the same time avoids damage to the photosystems with a high instantaneous photoprotective mechanism as assessed by VAZ pool size and DEPS. This response to drought is very efficient during shorter periods of drought, but might be a disadvantage during longer drought periods: Conrad Creek showed the highest drought sensitivity in 2003 among six coastal provenances as shown by the largest increase in IWUE and strongest decline in radial growth (Figure 15), and might thus be highly susceptible to carbon starvation during extreme drought.

In contrast to the strong stomatal and photoprotective response in Conrad Creek, monoterpene pools and emissions responded on the lowest level to temperature and sunshine hours in this provenance. Carbon costs of these protective mechanisms are apparently low in Conrad Creek, potentially contributing to the high growth potential under average conditions.

The provenances Timber and Santiam River both originate from regions with the lowest July to August precipitation among the provenances tested here (Figure 3) and the highest July Hargreaves climatic moisture deficit among the coastal provenances (Table 3). These two provenances were – among coastal provenances – least affected by the extreme drought event in 2003 as revealed by the smallest decline in radial growth and smallest increase in IWUE (Figure 15). Timber, originating from the Coast Range in Oregon, yielded intermediate to top

ranks in long term height growth at different elevations (Table 14). Thus, in this provenance we see a combination of high drought resistance and high growth potential under average conditions.

Despite originating from a region with lowest July and August precipitation (Figure 3), the provenance Duncan Paldi showed an intermediate decline in radial growth and intermediate increase in IWUE during the drought event in 2003 (Figure 15), similar to Pamelia Creek. Both provenances originate from a region with an intermediate Hargreaves climatic moisture deficit in July and August (CMD, Table 3). Thus, this parameter might give a better insight into the climatic conditions in the region of origin (and the adaptation of a provenance to drought) than precipitation sums. When correlating the increase in IWUE of six coastal provenances during the drought event in 2003 with monthly, mean annual and mean summer climatic parameters in the region of origin, we find the IWUE response to the drought in 2003 in Southern Germany related to August CMD as well as to July precipitation sum (R^2=0.48 and 0.50, respectively), though not significantly (p=0.12, data not shown). When excluding the provenance Duncan Paldi, we find a significant correlation with July precipitation sum in the region of origin (R^2=0.91, p=0.003) whereas the correlation with CMD remains stable. However, considering all climatic parameters and coastal provenances tested in this study, August CMD and July precipitation sum might give the most reliable insight into the climatic conditions in the region of origin and an estimate of the drought response of a provenance. Useful information might also be the frequency of severe droughts that do not occur regularly and are not reflected in long term average data.

However, the provenance Santiam River, though least affected by the extreme drought event in 2003 as revealed by alterations in IWUE and radial growth (Figure 15), showed the lowest long-term height growth (Table 14; Jansen *et al.*, 2013; Neophytou *et al.*, 2016) and the lowest assimilation rate under average conditions (appendix; Junker *et al.*, 2017). We thus see a trade-off between the response to extreme events and the growth potential under moderate environmental conditions in this provenance (for an overview see Table 15 & 16). A similar trade-off was also reported for lodgepole pine (Montwé et al., 2016) and Douglas-fir (Montwé et al., 2015) with the provenances from the most southern locations showing the highest drought resistance but low productivity (Montwé et al., 2016). Investments into safety mechanisms such as high proportions of late wood consisting of cavitation-resistant dense

wood (Hacke *et al.*, 2001) could contribute to growth limitations of the drought-resilient provenances. Barnard *et al.* (2011) reported adjustments in xylem efficiency, safety and water storage capacity in Douglas-fir and ponderosa pine growing along a gradient of increasing aridity east and west of the Cascade Mountain Range. Martinez-Meier *et al.* (2008) found higher survival of Douglas-fir trees with tree-rings of higher wood density, and Dalla-Salda *et al.* (2009, 2011) demonstrated genetic differences and a link between wood density, cavitation resistance, and growth performance of mature Douglas-fir clones under the severe drought event of 2003 in Europe. Investigating wood density and growth performance during severe drought in apparently drought resistant provenances such as Santiam River and others would give further insight into the mechanisms behind the drought resistance and growth potential. In the studies being part of the present thesis, we mainly focussed on leaf level traits linked to drought resistance.

Santiam River is a coastal Douglas-fir provenance from the Cascade Range in Oregon. Considering the monthly Hargreaves climatic moisture deficit in July and August (CMD=130 and 108 mm, Table 3), Santiam River originates from the driest region among all coastal and interior provenances studied here. The annual CMD is highest in Santiam River among the coastal provenances (346 mm, Table 2). Only the interior and the transition provenances show comparable or higher annual CMD (397 and 427 mm, respectively). The drought response in Santiam River might thus show an adaptation to a region of origin with intensive summer drought. The long-term height growth in Douglas-fir provenances, amongst them Santiam River, has recently been linked to the climate of origin and genotypic variation (Neophytou et al., 2016) but it remains to be studied if the physiological response to drought reflects genotypic adaptation to extreme drought periods.

Santiam River showed the smallest increase in IWUE in response to the extreme drought in 2003 (Figure 15). Furthermore, gas exchange measurements in 2010 and 2011 under moderate to dry conditions showed that A_n and g_s levels were sign. lowest in Santiam River but at the same time were least responsive towards water availability among four provenances (Figure 4 in Junker *et al.*, 2017). We conclude that both gas exchange and isotope analyses show a small operational range of stomata and a rather anisohydric regulation of g_s under drought and moderate conditions.

The adaptation to drought can entail enhanced osmotic adjustment (OA) as discussed in the previous chapter. Under dry conditions, OA by accumulation of inorganic ions was also most pronounced in Santiam River among the four provenances studied at the dry field site Wiesloch (chapter 2): Here, both leaf water content and leaf mineral content increased under drier conditions (Figure 9), and the increase was strongest in the provenance Santiam River (mineral content correlated with soil water availability, R^2=0.87, p=0.066). Moreover, both parameters (leaf water and mineral content) were significantly correlated to each other in Santiam River (R^2=0.31, p=0.003, Figure S 1) whereas in the other provenances this relation was not significant. OA regulates plant cell turgor and maintains leaf water content, stomatal conductance and photosynthesis under low soil water availability. OA can thus indirectly affect IWUE and $\delta^{13}C$ of plant organic matter. Under extreme drought we would thus expect increases in IWUE to be less pronounced in plants with enhanced OA. Our results for the provenance Santiam River point to a relationship between a rather low IWUE response to drought stress (chapter 3) and high osmotic adjustment (chapter 2).

The increase in monoterpene emissions in response to sunshine duration was highest in Santiam River (Figure 8B in Junker *et al.*, 2017), indicating a rapid instantaneous response mechanism to protect the trees from thermal and oxidative damage (Vickers et al., 2009). Furthermore, the biosynthesis of non-essential isoprenoids serves as a metabolic sink for electrons that result from the uptake of excess energy (Owen & Peñuelas, 2005). However, the emission of volatile isoprenoids may also contribute to a loss of previously fixed carbon (Simpraga *et al.*, 2011; Ryan *et al.*, 2014) and thus to the low height growth potential observed in this provenance (chapter 2; Jansen *et al.*, 2013; Neophytou *et al.*, 2016). Furthermore, Santiam River showed the lowest assimilation rates among four provenances (Junker *et al.*, 2017), further depriving the height growth potential.

Lowest xanthophyll cycle pigments pool size, high chlorophyll content under drought and low carotenoid-chlorophyll ratio (Figure 6 and 7 in Junker *et al.*, 2017) point to a comparably low photoprotective capacity in Santiam River. With an anisohydric regulation of water status, the photosynthetic CO_2 fixation under drought might be less impaired, the formation of reactive oxygen species less supported and the photoprotective capacity might have lower priority than in provenances with isohydric behaviour.

In summary, the provenance Santiam River from the most southern and relatively dry region of origin studied in our field experiment (driest among the coastal provenances; see Table 2) showed an anisohydric regulation of stomatal conductance, high levels of osmotic adjustment and highest monoterpene emissions under dry conditions together with lowest long term height growth under average conditions at South German field sites. Our results might reflect genotypic adaptation to dry and warm environments in Santiam River.

Table 15 Summary on gas exchange parameters (GE), growth and stress response mechanisms assessed in four provenances under average water supply
The signs indicate lowest (---), low (-), medium (~), high (+) and highest (+++) level, or positive (+, +++), negative (-, ---) and no response (0)

Provenance Var.	Salmon Arm interior	Santiam River coastal	Conrad Creek coastal	Cameron Lake coastal	Reference
Gas exchange under average water supply					
IWUE derived from leaf isotopic composition	---	~	+	+++ (Schluchsee) ~ (Wiesloch)	Chapter 2
An derived from GE	+++ (Schluchsee) + (n.s., Wiesloch)	---	+	+	Appendix (Junker et al., 2017)
Growth under average water supply					
Long term height growth under average conditions	---	-	+ / +++	variable -/+/+++	Chapter 3 Neophytou 2016
VOC under average water supply					
Monoterpene pools	+++	-	-	-	Appendix (Junker et al., 2017)
Summary	Low height growth despite medium to high An C allocation more towards secondary metabolism (e.g. monoterpenes) and less towards growth	Low height growth linked to lowest An	High height growth linked to high An C allocation towards growth	High height growth linked to high An C allocation towards growth	

Table 16 Summary on gas exchange parameters (GE), growth and stress response mechanisms assessed in four provenances under drought conditions
The signs indicate lowest (---), low (-), medium (~), high (+) and highest (+++) level, or positive (+, +++), negative (-, ---) and no response (0)

Provenance Var.	Salmon Arm interior	Santiam River coastal	Conrad Creek coastal	Cameron Lake coastal	Reference
Gas exchange under drought					
IWUE response to extreme drought in 2003 derived from tree ring isotopic composition	NA	0/+	+++	+	Chapter 3
g_s response to drought (dual isotope approach in tree rings)	NA	0 no stomatal control on ci anisohydric	- isohydric	- isohydric	Chapter 3
gs response to drought (GE)	- isohydric at Schluchsee -/0 intermediate at Wiesloch	0 nearly no response anisohydric	--- isohydric	- isohydric	Chapter 2 Appendix (Junker et al., 2017)
Growth under drought					
Radial growth response to drought	NA	-/0	---	-	Chapter 3
VOC under drought					
Monoterpene emission under drought	+	+++	+	+	Appendix (Junker et al., 2017)
Photoprotection under drought					
VAZ response to drought	+	0	+	+++	Appendix (Junker et al., 2017)
ß car / chl ratio response to drought	0/+	+++	+++	+	Appendix (Junker et al., 2017)
Summary	Small response to drought in terms of stomatal closure Medium photoprotection Medium monoterpene emission under drought	Smallest response to drought in terms of stomatal closure & growth decline Low instantaneous but high adaptive photoprotection Highest monoterpene emission under drought	Strongest response to drought in terms of stomatal closure & growth decline High photoprotection Medium monoterpene emission under drought	Medium response to drought in terms of stomatal closure & growth decline High photoprotection Medium monoterpene emission under drought	

Cameron Lake, a coastal provenance from Vancouver Island in British Columbia (Canada), showed highest levels of g_s during average and dry periods at Wiesloch (Junker *et al.*, 2017) but also the highest range in g_s at this site pointing to a rather isohydric regulation of g_s. This also explains the high responsiveness of assimilation rate to water availability in this provenance (Figure 4 in Junker *et al.*, 2017).

Long term height growth in Cameron Lake was extremely site-dependant, ranging between lowest and highest ranks among four provenances (Table 14). The growth decline in the extreme dry and hot year 2003 was intermediate in the provenance Cameron Lake (Figure 15). This provenance displays an intermediate drought sensitivity and long term height growth potential under average conditions.

With highest xanthophyll cycle pigments (VAZ) pool size and a strong response of VAZ pools to daily sunshine hours (R^2=0.52; Figure 7A in Junker *et al.*, 2017), Cameron Lake shows a high photoprotective capacity. A strong positive relation between the de-epoxidation state of the xanthophyll cycle pigments (DEPS) and sunshine hours was found in all studied coastal provenances, especially in Cameron Lake (R^2=0.82), indicating an instantaneous mechanism to dissipate excess light energy as heat and facilitate NPQ (Niyogi, 2000; Demmig-Adams & Adams, 2006). However, high chlorophyll content and rather low carotenoid-chlorophyll ratio under drought as found in Cameron Lake (Figure 6 in Junker *et al.*, 2017) have been attributed to low levels of photoprotection (Duan *et al.*, 2005; Baquedano & Castillo, 2006; Gallé *et al.*, 2007). β-carotene, protecting the reaction centres of the photosystems 32, was intermediate in LA (Figure 3 and Table 4 in Junker *et al.* (Junker *et al.*, 2017)). The pigment composition of the photosynthetic apparatus, adjusted on longer timescales, is a species-specific trait developed during adaptation to environmental conditions (Ensminger *et al.*, 2004; Croce & van Amerongen, 2014; Fréchette *et al.*, 2015). The pigment composition in Cameron Lake thus points to an adaptation to a rather humid region of origin. Despite of this, Cameron Lake shows a high level of instantaneous NPQ via adjustments in the VAZ pools, pointing to a high plasticity in the response to excess energy during drought.

Monoterpene pools are small in all coastal provenances when compared to the interior provenance. However, monoterpene pool size increases with temperature in Cameron Lake (R^2=0.62; Figure 8 in Junker *et al.*, 2017) and might be linked to high gene expression levels as observed by Hess et al. (2016): Transcripts related to isoprenoid biosynthesis and metabolism were highly expressed in Cameron Lake. The positive temperature response in monoterpene pools of Cameron Lake is in strong contrast to the negative response in the other coastal provenances. At the same time, emissions of monoterpenes are on a very low level in Cameron Lake. Thus, among the coastal provenances, Cameron Lake shows a comparably large heat protective potential without emitting large portions of its monoterpene pools. The opposite

pattern was found in Santiam River with a decreasing monoterpene pool size under increasing temperature and increasing monoterpene emissions.

In Cameron Lake, the extremely site-dependant long term height growth (highest under humid conditions), a rather isohydric regulation of g_s and the photosynthetic pigment composition point to an adaptation to rather humid growing conditions. Nevertheless, high levels of instantaneous NPQ and the responsiveness in monoterpene pool sizes show certain plasticity in the response to drought and heat in Cameron Lake.

5.2.2 Interior and transition Douglas-fir provenances

In contrast to the coastal provenances discussed before, Salmon Arm originates from the *menziesii-glauca* transition zone in southern interior British Columbia, with a mean annual precipitation sum of 362-415mm (Table 2) the driest environment of origin assessed here. Genetic analyses revealed a significant differentiation of Salmon Arm from the coastal provenances (Müller et al., 2015; Neophytou et al., 2016) strongly indicating that Salmon Arm belongs to the interior variety. These also showed that individuals of Salmon Arm form a distinct subpopulation (Neophytou et al., 2016), confirming that the source population is located in an area where the coastal and interior variety may intermingle due to postglacial recolonization of Canada by both varieties (Little, 1971; Sorensen, 1979; Gugger *et al.*, 2010). However, our results on physiological responses towards average to dry conditions (chapter 2 and appendix; Junker *et al.*, 2017) showed again a clear distinction between coastal provenances and Salmon Arm that we refer to as interior provenance.

Intrinsic water-use efficiency assessed by leaf carbon isotope composition (integrating over one growing season) was lowest in Salmon Arm at Schluchsee (chapter 2), which we associated to extraordinary high levels of g_s as assessed by Junker *et al.* (Junker *et al.*, 2017). During humid periods at Schluchsee, g_s was significantly highest in this provenance from the driest region of origin. We made a similar observation in our study on tree rings from 2002 to 2007 (chapter 3) when averaging isotopic data over the 6-years period at the valley site Mooswald: The combined $\delta^{13}C$ and $\delta^{18}O$ data and their opposite correlation with temperature and precipitation at the sites of origin (Table 12) point to the fact that under average conditions the stomata are likely to be more open (lower $\delta^{13}C$ and $\delta^{18}O$) in provenances originating from warmer and drier regions compared to those from cooler and wetter regions (higher $\delta^{13}C$

and $\delta^{18}O$). We might conclude from both studies that provenances from drier regions of origin respond to average and humid conditions with enhanced g_s.

However, interior provenances have been consistently reported to have less growth potential (see meta-analysis of Isaac-Renton *et al.* 2014) but higher drought resistance (Ferrell & Woodard, 1966; Pharis & Ferrell, 1966; Kavanagh *et al.*, 1999) and higher cold resistance than the coastal variety (e.g. Rehfeldt, 1977). Our results showing enhanced g_s under humid conditions and enhanced assimilation rates under both humid and dry conditions (Junker *et al.*, 2017) seem to contradict this, as high gas exchange should allow for a high growth potential. Indeed, a mismatch between high assimilation rates and low growth became apparent as Salmon Arm showed the lowest long term height growth at Wiesloch and Schluchsee, and medium height growth at the field site Sindelfingen (Neophytou *et al.*, 2016).

A large growth reduction under severe drought was revealed in Southwest France (Sergent et al., 2014) in the southern interior provenances in 2004, the second successive year of drought in this region, whereas coastal provenances exhibited a comparably small growth reduction. These surprising results might be linked to differences in stomatal regulation of leaf water status and associated risk of hydraulic failure under exceptionally severe droughts. Similarly, during a record drought in the south eastern USA, species with high wood density, cavitation resistance and stomatal conductance displayed higher levels of native embolism and greater canopy dieback than species with low wood density that avoided catastrophic embolism by relying on partial drought deciduousness and higher sensitivity of stomata to leaf water potential (Hoffmann *et al.*, 2011).

The observed mismatch between low growth potential in Salmon Arm and high assimilation rates might be explained by other, potentially metabolic, processes and their carbon costs (see overview in Table 15 & 16): Some metabolic pathways are strongly upregulated in this provenance, for instance monoterpene pools were by far highest in Salmon Arm compared to the coastal provenances (Figure 8A in Junker *et al.*, 2017; appendix), pointing to the antioxidant and heat protective potential of Salmon Arm. Furthermore, Monte Creek and Salmon Arm originate in the *menziesii-glauca* transition zone in southern interior British Columbia, the driest environment in the natural range of Douglas-fir. Both showed enhanced activity of the phenylpropanoid metabolism: Levels of Quinic acid, linked to phenylpropanoid synthesis (Macheix *et al.*, 1990; Bennett & Wallsgrove, 1994), drought stress and antioxidant activity

(Rivas-Ubach *et al.*, 2012; Yang *et al.*, 2013), were highly abundant and significantly enhanced in 2-year old seedlings of Monte Creek under increased heat and atmospheric drought (chapter 4). Hess et al. (2016) reported higher gene expression levels of the phenylpropanoid metabolic processes in mature trees of the provenance Salmon Arm when compared to the coastal provenance Cameron Lake. Thus, gene expression as well as metabolic analyses revealed the upregulation of specific metabolic pathways in provenances from Southern Interior British Columbia. Further research might reveal whether the upregulation of this specific metabolic process is on the one hand linked to genotypic adaptation or not and might on the other hand be responsible for less carbon being allocated to growth.

Furthermore, the pigment composition of the photosynthetic apparatus points to an adaptation to rather dry conditions as it is adjusted on long timescales (Ensminger et al., 2004; Croce & van Amerongen, 2014; Fréchette et al., 2015). Salmon Arm displayed an increased long term adjusted photoprotective capacity as shown by significantly lowest levels of chlorophyll (c.f. Duan *et al.*, 2005; Baquedano & Castillo, 2006; Gallé *et al.*, 2007), highest carotenoid per chlorophyll ratios and highest β-carotene per chlorophyll ratios under drought (Figure 3, Figure 6 and Table 4 in Junker *et al.* (Junker *et al.*, 2017)). These photoprotective mechanisms potentially contribute to the adaptation to rather dry conditions and the high assimilation rates observed in this provenance.

In contrast, instantaneous photoprotective mechanisms were less pronounced in Salmon Arm (Figure 7 in Junker *et al.*, 2017; appendix). Gas exchange in Salmon Arm was medium to high under various environmental conditions (Junker *et al.*, 2017), suggesting that damage to the photosystems is less likely in Salmon Arm and instantaneous photoprotective mechanisms might not be associated to the adaptation to frequent drought.

Bansal *et al.* (2014) suggested that populations from regions with relatively cool winters and arid summers are potentially best adapted to warm and dry conditions. We conclude from the results discussed in this section that a high antioxidant, (long term adjusted) photoprotective, drought and heat protective potential most probably provides an advantage under very dry and hot conditions at the expense of growth in provenances from Southern Interior British Columbia such as Salmon Arm.

In an experiment under controlled conditions (see chapter 4 and appendix; Du *et al.*, 2014) we applied a combined treatment of heat and atmospheric drought to tree saplings of two provenances originating from dry regions of origin (Table 2). Monte Creek is, as the interior Salmon Arm, a Douglas-fir provenance from the *menziesii-glauca* transition zone in southern interior British Columbia, whereas the interior provenance Pend Oreille originates from the *glauca* zone in Northeast Washington State (392 and 736 mm mean annual precipitation, 433 and 397 mm annual climatic moisture deficit, respectively).

Du *et al.* (2014, see Appendix) noted some clear differences between Pend Oreille and Monte Creek in nitrogen (N) partitioning in response to increased temperature and atmospheric drought. Pend Oreille originating from a more humid environment showed reduced contents of N-containing compounds, most notably of amino acids involved in the transport and storage of reduced N. This suggests that the N status of this provenance was negatively affected by elevated temperature.

In chapter 4 we discussed further differences of the two provenances in their metabolic response regarding osmotic adjustment, respiratory fractionation and substrate commitment towards specific pathways, where a parallel trend became apparent though. The more negative apparent respiratory fractionation under elevated temperature (decrease by 2.2% for Pend Oreille and 3.0% for Monte Creek, Figure 17) indicated a temperature-dependent switch in the substrate commitment from the tricarboxylic acid cycle to other metabolic pathways. Metabolic analyses in both provenances showed a significant increase in the total amount of quinic acid which is linked to phenylpropanoid synthesis, plant defence, drought stress, and antioxidant activity.

Osmoprotective and ROS scavenging polyols increased in both provenances whereas proline decreased (see chapter 4 and Appendix; Du *et al.*, 2014). Proline has been shown to be less efficient in osmoprotection at high temperatures (Smirnoff & Stewart, 1985), and in ROS scavenging (Buxton *et al.*, 1988) compared to polyols. We therefore conclude that there is a preference for the trees to produce polyols at the expense of proline synthesis under high temperature. This preference was most pronounced in Pend Oreille. The observed accumulation of monosaccharides might also reflect their role in a heat stress-specific replacement for proline as an osmoprotectant (Rizhsky *et al.*, 2004).

In summary, under high temperature and atmospheric drought, an array of metabolites protecting against high temperature, osmotic stress, and reactive oxygen species was accumulated in both provenances, whereas total biomass (Figure S 6) and plant $\delta^{13}C$, as a marker for intrinsic water-use efficiency (Figure 16), were largely unaffected. The two provenances from the *menziesii-glauca* transition zone and the *glauca* zone thus demonstrated the physiological plasticity that allows continued assimilation and growth during extended periods of high temperatures.

Some observations such as the preference for the trees to produce polyols at the expense of proline synthesis under high temperature (chapter 4), or reduced contents of nitrogen-containing compounds under high temperature (Du *et al.*, 2014, see Appendix), were most pronounced in the provenance originating from a more mesic environment (Pend Oreille). These results indicate that this provenance is more heat sensitive and might experience even stronger N deficiency if grown under N-limiting conditions.

6 Conclusion

In field and controlled experiments, we applied and compared different methodological approaches to reveal physiological responses of Douglas-fir provenances towards heat and drought. As described in chapter 4, both the apparent respiratory fractionation and metabolite profiles showed a metabolic switch towards the synthesis of secondary and heat protecting compounds. Isotopic composition of leaf TOM (integrating up to the whole lifespan of the leaf), WSOM (as a proxy for recent assimilates; (Gessler *et al.*, 2009c), as well as instantaneous gas exchange measurements of A_n and g_s, revealed concurring results for the intrinsic water-use efficiency in adult Douglas-fir trees on a humid field site (chapter 2). Our results at the dry field site showed a severely limited operational range of stomata, partly explaining the weak relation between IWUE derived from different methods. Furthermore, osmotic adjustment was much stronger at the dry field site while leaf $\delta^{13}C$ was not correlated to water availability there. Thus, under generally dry conditions over the long-term, we need to take into account the effect of osmotic regulation on leaf physiology so that the relationship between isotopic composition and weather parameters might not be interpreted straightforward.

Douglas-fir provenances displayed contrasting strategies to cope with environmental stress, ranging from isohydric regulation of water status with stomatal closure under drought and high instantaneous photoprotective mechanisms (Conrad Creek), anisohydric regulation with weak stomatal responses to drought under high osmotic adjustment and monoterpene emissions (Santiam River), and medium stomatal responses combined with generally high gas exchange but high monoterpene pools (Salmon Arm). Cameron Lake displayed intermediate drought sensitivity and high photoprotective capacity, with the highest responsiveness of stomatal conductance and assimilation rate to water availability.

Some provenances show a trade-off between the response to extreme events and the growth potential under moderate environmental conditions: The coastal provenance Santiam River showed the lowest long-term height growth but was least affected by the extreme drought event in 2003 as revealed by alterations in IWUE and radial growth. In contrast, Conrad Creek showed the highest long-term height growth but was most affected by the extreme drought event in 2003. Cameron Lake showed a similar physiological response as Conrad Creek but an extremely site-dependant long term height growth under average conditions. The

provenance Monte Creek, with an origin very similar to that of Salmon Arm in southern interior British Columbia, showed a high resistance towards heat and atmospheric drought. Salmon Arm displayed only average long term height growth despite high assimilation rates under various environmental conditions which can be partly explained by high metabolic costs of monoterpene pools. The coastal provenance Timber, however, displayed a combination of high drought resistance in terms of radial growth decline and stomatal response, and comparably high long term growth potential under average conditions. The observed long term height growth of Douglas-fir provenances reflects their growth potential under average conditions which will – to a smaller or larger extent – not match future conditions, depending on regional or local site conditions. Foresters focusing on provenances with maximum growth potential under average conditions only might not achieve economically optimal results in future decades. Instead, a mixture with drought tolerant provenances should be considered. (Bansal et al., 2014) suggested that populations from regions with relatively cool winters and arid summers (such as Salmon Arm and Monte Creek) are potentially best adapted to warm and dry conditions. We might conclude from our results that a high antioxidant, photoprotective, drought and heat protective potential most probably provides an advantage under very dry and hot conditions at the expense of growth in provenances from Southern Interior British Columbia. In regions with a prospected high risk of increasing heat and drought, these provenances might provide an advantage in forest management. Further research on these provenances should also examine the resilience and the recovery of trees during post-stress years to assess more long term effects of extreme events on tree vitality.

Among the coastal provenances, we observed a low drought response in provenances originating from regions with a high Hargreaves climatic moisture deficit and low precipitation sum in July and August. These climatic parameters, rather than mean annual or summer parameters, might give a better insight into the climatic conditions in the region of origin and into the potential adaption of provenances to dry conditions. Furthermore, the highly diverse growth potential under average conditions of the provenances showing a low drought response demonstrated that there can be a trade-off between the response to extreme events and the growth potential under moderate environmental conditions but there is also the potential of high drought resistance combined with medium to high long term growth potential under average conditions which should be of high interest for forest management.

In summary, Douglas-fir shows remarkable drought stress resistance in Central Europe and can thus, under economic points of view, be considered a replacement species for spruce. We observed large differences in the provenances' stress response mechanisms that should be considered especially with regard to the environmental conditions prevailing (and expected in the future) at the respective growing sites.

Appendix

Co-authored Publications

Elevated temperature differently affects foliar nitrogen partitioning in seedlings of diverse Douglas fir provenances

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Variation in short-term and long-term responses of photosynthesis and isoprenoid-mediated photoprotection to soil water availability in four Douglas-fir provenances

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Author's Declaration

Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbstständig und ausschließlich unter Verwendung der angegebenen Quellen sowie ohne unzulässige Hilfe Dritter angefertigt habe. Diese Doktorarbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form oder auszugsweise einer Prüfungsbehörde vorgelegt.

……., den …….

Kirstin Jansen

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